University of São Paulo
"Luiz de Queiroz" College of Agriculture

Soil microbiome manipulation and its effect on the soil-plant interface

Bruna Arruda

Thesis presented to obtain the degree of Doctor in Science. Area: Soil and Plant Nutrition

Piracicaba
2020

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Agronomist

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
Prof. Dr. **FERNANDO DINI ANDREOTE**

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1. Fósforo 2. Nitrogênio 3. *Urochloa brizantha* cv. Marandu 4. *Crotalaria juncea* L. I. Título

To my grandparents Celencina & Livino (in memoriam) and Hilda & Milton

To my parents, Catarina & Edu

To my brother Vitor

I dedicate

Aos meus avós Celencina & Livino (in memoriam) e Hilda & Milton

Aos meus pais, Catarina & Edu

Ao meu irmão, Vitor

Dedico

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“Don't wait for extraordinary opportunities.

Seize common occasions and make them great.

Weak men wait for opportunities; strong men make them.”

Orison Swett Marden

CONTENTS

RESUMO

Manipulação do microbioma do solo e seu efeito na interface solo-planta

O objetivo deste projeto foi avaliar o efeito da manipulação do microbioma do solo nas relações entre os microrganismo com solo e planta. Dentre estas relações destacam-se as micorrizas. Muitos fatores são determinantes para sua ocorrência, dentre eles a comunidade microbiana do solo. Nossos resultados mostraram que o efeito da comunidade microbiana do solo na micorrização depende da espécie vegetal. A colonização micorrízica em plantas de braquiária foi prejudicada pela manipulação do microbioma, porém determinadas espécies de fungos micorrizicos arbusculares foram capazes de colonizar crotalária mesmo sob solo com comunidade microbiana reduzida. Para determinar as taxas de colonização micorrízica atualmente é utilizada a microscopia, porém trata-se de uma análise subjetiva. Para elimiar esse viés, técnicas moleculares, como a PCR em tempo real (qPCR), podem ser uma alternativa para padronizar essa avaliação. Nossos resultados mostraram que o conjunto de primers FLR3 e FLR4 apresentou correlação de 0,73 entre as análises microscópica e de qPCR, sendo portanto uma alternativa promissora para analises de micorriza. Além dos microganismos do solo que auxiliam as plantas na absorção de P, a aplicação de fertilizantes fosfatados é uma prática utilizada para fornecer este nutriente. Entretanto constatamos que a aplicação de P não compensou o efeito da alta competição entre planta e microorganimos do solo pelo P. Braquiária apresentou maior absorção de P e acúmulo de matéria seca da parte aérea quando cultivada sob solo com diversidade microbiana reduzida. Por outro lado, a crotalária mostrou um efeito neutro para a manipulação microbiana. Estudos que envolvem a manipulação microbiana do solo podem utilizar técnicas como a autoclavagem de solo. Esta técnica utiliza alta temperatura e pressão que em conjunto eliminam grande parte dos microrganismos, o que pode também afetar a química do solo. Nossos resultados mostraram que, em solo autoclavado a emissão de $^{14}CO_2$ via respiração microbiana foi nula e não houve alterações no N total em relação ao solo natural, mas houve um aumento no $N\text{-}NH_4$ em relação ao $N\text{-}NO_3$. Além disso, o procedimento de autoclavagem não alterou a concentração de ^{31}P do solo, mas reduziu a atividade do ^{33}P lábil. Estudos sobre respostas de diferentes espécies de plantas cultivadas em solos com diferentes níves de diversidade microbiana do solo são necessários para o entendimento da ciclagem de nutrientes como N e P. Nossos resultados mostraram que baquiaria aumentou a produção de massa seca, absorção de N e P quando cultivada sob solo que sofreu redução do microbioma durante o 1º ciclo da planta. Para o 2º ciclo, braquiaria aumentou a absorção de N, mas sem aumento na produção de massa seca da parte aérea quando cultivada em solo manipulado em comparação ao solo sem manipulação microbiana. Por outro lado, a crotalária não foi afetada pela manipulação do microbioma para a matéria seca da parte aérea, absorção de N e P. Assim, os resultados dessa tese podem auxiliar no manejo de áreas biologicamente degradadas, para garantir uma maior sustentabilidade nesses sistemas.

Palavras-chave: Fósforo, Nitrogênio, *Urochloa brizantha* cv. Marandu, *Crotalaria juncea* L.

ABSTRACT

Soil microbiome manipulation and its effect on the soil-plant interface

The aim of this project was to evaluate the effect of soil microbiome manipulation on microorganism relationship with soil and plant. Among these relationships are mycorrhizae. Many factors are determinant for its occurrence, including the soil microbial community. Our results showed that the effect of soil microbial community on mycorrhization depends on the plant species. Mycorrization in brachiaria was impaired by microbiome manipulation, but certain species of arbuscular mycorrhizal fungi were able to colonize crotalaria even under soil with reduced microbial community. To determine mycorrhizal colonization rates, microscopy is currently used, but it is a subjective analysis. To eliminate this issue, molecular techniques such as real-time PCR (qPCR) may be an alternative to standardize this assessment. Our results showed that the set of primers FLR3 and FLR4 showed a correlation of 0.73 between the microscopic and qPCR analyzes, thus being a promising alternative for mycorrhiza analyzes. In addition to soil microorganisms that assist plants in the absorption of P, the application of phosphate fertilizers is a practice used to supply this nutrient. However, we observed that the application of P did not compensate for the effect of high competition between plant and soil microorganisms by P. Brachiaria presented higher P uptake and dry matter accumulation when cultivated under soil with reduced microbial diversity. On the other hand, crotalaria showed a neutral effect for microbial manipulation. Studies involving soil microbial manipulation may use techniques such as soil autoclaving. This technique uses high temperature and pressure that together eliminate most of the microorganisms, which can also affect soil chemistry. Our results showed that in autoclaved soil $^{14}CO_2$ emission via microbial respiration was null and there was no change in total N compared to natural soil, but there was an increase in $N\text{-}NH_4$ compared to $N\text{-}NO_3$. In addition, the autoclaving procedure did not alter soil ^{31}P concentration, but reduced labile ^{33}P activity. Studies on responses of different species of plants grown in soils with different levels of soil microbial diversity are needed to understand nutrient cycling such as N and P. Our results showed that bachiaria increased dry matter, N and P uptake. when cultivated under soil that suffered microbiome reduction during the 1st cycle of the plant. For the 2nd cycle, brachiaria increased N uptake but no increments in shoot dry mass when grown in manipulated soil compared to soil without microbial manipulation. On the other hand, crotalaria was not affected by microbiome manipulation for shoot dry matter, N and P absorption. Thus, the results of this thesis may help in the management of biologically degraded areas to ensure greater sustainability in these systems.

Keywords: Phosphorus, Nitrogen, *Urochloa brizantha* cv. Marandu, *Crotalaria juncea* L.

1 INTRODUCTION

To guarantee the agriculture production, there are many factor that must be taken into account. A problem that is increasing in last years is the degradation of the agriculture area due to the inappropriate management of the soil affecting the biological, physical, chemical and ecological relationships. Losses of the microbial diversity and chemical fertility may harm the agriculture production. Soil microrganisms are vital because they participate in many process and can interact with plants, providing nutrient to the crops. The nutrients are required by the plants to complete their cycle and keep the yield, and are essential for agriculture production.

The aim of this project was to evaluate the effect of soil microbiome manipulation on microorganism relationship with soil and plant. In order to achieve it, we developed five experimental chapters.

Among the relationships that occur between microorganisms and plants, we can remark mycorrhiza association. This association occurs between arbuscular mycorrhizal fungi (AMF) and most of the vascular plants. Through this association the fungi colonize the root and the hyphae are capable to increase the exploration of the soil, therefore promoting the absorption of nutrients by the plant, mainly the ones with low mobility in the soil, such as P. Many factors are determinant for this relationship to occur, among them the soil microbial community, such as the mycorrhiza helper bacteria (MHB). These organisms are described to assist the colonization of the roots by the AMF. However, little is known about how much soil microbial diversity levels affect the association between different mycorrhizal fungi species and different plant species, mainly due to the interspecificity feature. We run an experiment in glasshouse to compare the mycorrhiza colonization in brachiaria, a grass, and crotalaria, a legume, cultivated in soil with levels of microbial diversity and inoculation of different AMF species.

To determine the rates of mycorrhizal colonization, currently, the standard procedure used is evaluation in microscope. This evaluation require knowledge and experience of the evaluator, and so the analysis is quite subjective, which may harm the results. To mitigate this issue molecular techniques, such as real-time PCR (qPCR), may be an alternative to facilitate the standardization of this evaluation, and studies on their practical application are required. We correlated both microscope and qPCR tecniques using root samples from brachiaria and crotalaria.

In addition to the soil microrganism to assist plant on P uptake, a practice of applying phosphate fertilizers is used to provide this nutrient to the crops. The main source of

phosphate is apatite, which is finete resource and its usage need to be with caution to guarantee the food production for the next generation. We run an experiment in glasshouse to understand the compensatory effect between level of microbial diversity and P application via fertilizers on the P uptaken response and dry matter production of different plant species, and so, if the application of P fertilizer would compensate the changes of microbial diversity community.

For microbial diversity studies, many methods can be used for removing organisms from the soil. One of the simplest techniques is soil autoclaving. This technique uses high temperature and pressure that eliminate most of the microorganisms. However, due to autoclaving temperature and pressure conditions, chemical changes may occur and studies of the effects of autoclaving on soil chemical features are required. This is because C, N and P can be affected by autoclaving itself or the loss of microorganisms that participate in the cycling of these nutrients. To measure the effect of the autoclaving in the soil on microbial activity and P and N changes we run an experiment in laboratory, under controled condition.

Additionaly, studies on responses of different plant species grown in soils with different soil microbial commnunities are needed. For this, we run an experiment in glasshouse using brachiaria and crotalaria for two crop cycles to understand nutrient cycling such as N and P.

The hypothesis of this study was that changes in microbial community affect the interaction between soil and plant.

2 MYCORRHIZA COLONIZATION OF PLANTS UNDER MANIPULATED SOIL MICROBIOMES

Abstract
Phosphorus (P) has low mobility in the soil and the symbiotic relationship between roots and arbuscular mycorrhiza fungi (AMF) improves ion-root contact to promote the P uptake due to the soil exploration of the fungal hypha. The whole soil microbiome can interact with AMF population, affecting plant roots colonization. The objective of this study was to evaluate the mycorrhiza colonization of plants cultivated in a soil hosting distinct microbiomes generated by heat shock or natural community dilution. The experiment was conducted in a glasshouse, using soils treated with eight distinct compositions of the native microbiome: natural soil (i); soil exposed to heat treatments for one hour of 50 ºC (ii), 80 ºC (iii) or 100 ºC (iv); and soils autoclaved twice (121 ºC, 103 kPa, 1 h) to generate the sterilized soil (SS) followed by inoculation of dilutions (10% v/w of natural soil): SS+10^{-1} (v); SS+10^{-3} (vi); SS+10^{-6} (vii); SS (without dilution inoculation) (viii). The soil moisture was maintained at 70% of maximum hold capacity until the stabilization of the microbial community. Microbial (bacteria and fungi) community were assessed through ribosomal marker gene sequencing and the determination of the acid phosphatase activity (AP_{ASE}). Sequencing results showed distinct groups formed according to the methods used to manipulate the soil microbiome community and AP_{ASE} activity declined along stress gradient. After soil microbial community stabilization, seedlings of brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*Crotalaria juncea* L.) were transplanted to soils with separately inoculations of three AMF species (*Acalouspora colombiana*, *Rhizophagus clarus* or *Dentiscutata heterogama*), which were compared to an AMF-free control. We found that 30 days after AMF inoculation brachiaria mycorrhization was affected by the microbiome manipulation, showing higher colonization in natural soil when compared to other manipulated systems, regardless of the AMF species inoculated. Conversely, *A. colombiana* and *D. heterogama* were able to colonise crotalaria in similar rates to natural soil under modified microbial community, but *R. clarus* inoculation did not promote mycorrhization in crotalaria under manipulated systems. In summary, our results suggest that the grass was more dependent of the soil microbiome to promote root AMF colonization and the legume relied on the AMF species inoculated to colonize the roots.

Keywords: Microbiome manipulation; Microbial interaction; Fungi-bacteria interaction; Sterilized soil.

2.1 Introduction

Phosphorus (P) is an essential macronutrient required by all agricultural crops. It is characterized by its low mobility in weathered tropical soils, forcing plants to developed strategies that facilitate the ion-root contact. One of the most successful P acquisition strategies is the association with arbuscular mycorrhiza fungi (AMF), which can increase the volume of soil explored by the plant, making use of the fungal hyphae network (Machado and Furlani 2004).

Many factors regulate the occurrence of AMF and plants associations. One of the most important is the microbial community diversity present in the rhizosphere, which may

have a strong influence over plant-AMF interaction. This connection has been demonstrated, for example, through the interactions between AMF and the mycorrhiza helper bacteria (MHB), defined by Garbaye (1994) as the bacteria associated with mycorrhizal roots and mycorrhizal fungi which selectively promote the establishment of mycorrhizal symbioses.

In a review of the potential of MHB as plant growth promoting rhizobacteria (PGPR), Bruzos (2017) suggested mechanisms of symbiosis include: germination of fungal propagules, promotion of mycelial growth, modification of the mycorrhizosphere soil, host recognition and modifications in root system architecture, and receptivity of the roots. In this regard, Hestrin et al. (2019) noticed that there is synergism between the soil microbial community and AMF, which increased N uptake in *Brachypodium distachyon*. However, Svenningsen et al. (2018) observed a suppression effect on the AMF activity, caused by the soil microbiota, and concluded that this reduction is most likely due to the combined action of several members of the soil microbial communities. Therefore, the modification of the microbiome community and diversity may either promote or inhibit the mycorrhiza colonization activity.

Under controlled laboratory conditions, it is possible to manipulate the microbial community within the soil; for example, by extinction methods. These methods involve changes in soil features, such as pH or submit the soil to heat treatments (Voort et al. 2016), which may disrupt the microbial community, and create new niches where resistant and resilient organisms will establish. Another common method is the dilution of natural communities and further inoculation of aliquots in a sterile soil (van Elsas et al. 2012). In this case, changes are promoted by the reduction of microbial groups occurring in lower abundance in the soil. Thus, either heat shock or dilutions treatment can modify the microbial composition of the soil, possibly interfering in key functions in the soil system. Once the microbial community is modified, the interactions between its constituents are prone to change, such as interference in mycorrhiza colonization.

We posited that colonization would be dependent of specific interactions between plants, mycorrhizal fungi species and the soil microbiome constituents. To test this, we selected a grass and a legume, considering that theses plant groups are morphological and physiological distinct, and may pose differential selection upon the targeted process. In this sense, we evaluated the colonization of a grass and a legume, inoculated with spores from those three different groups, classified by Chagnon et al. (2013).

The objectives of this study were: i) to verify the extent of changes in soil microbiome caused by two diversity extinction methods (heat shock and dilutions); ii) to evaluate the

interference of these changes in the colonization of three AMF species in two distinct plants (grass and legume).

2.2 Material and methods

2.2.1 Experimental setup

The experiment was conducted in a glasshouse (27 °C ± 3), in factorial arrangement (8 x 4) and randomized block design, with four replicates. Each experimental unit comprised a plastic pot (5 cm internal diameter × 9 cm tall), containing 270 g of soil (dry weight). Soil was classified as a sandy clay loam textured Ferralsol (WRB-FAO, 2015), collected from a depth of 0-20 cm within a livestock-grazed pasture, in Piracicaba, Sao Paulo, Brazil (22°43'03.0''S, 47°37'00.4''W). The main characteristics of this soil are presented in Table 1 as natural soil (NS).

2.2.2 Soil microbiome manipulations

The soil was treated to generate eight different microbiomes. The first treatment, i) NS, was untreated and used as a positive control against the other treatments. To eliminate components of the microbial biomass, three separate microbiome treatments were generated by the heat the soil as follows: ii) 50 ºC for 1 h (S50), iii) 80 ºC for 1 h (S80); iv) 100 ºC for 1 h (S100). This method has been proved to generate a gradient of the natural soil microbiome along a temperature increase (Voort et al. 2016). In addition, four treatments were obtained by first sterilizing the soil by autoclaving twice (121 ºC, 103 kPa, 1 h). After 7 days, aliquots were re-inoculated using a dilution-to-extinction method in the sterile soil (van Elsas et al. 2012). This approach generated the treatments v) $SS+10^{-1}$ (10% v/w of natural soil dilution added to the sterile soil); vi) $SS+10^{-3}$; vii) $SS+10^{-6}$; and viii) sterile soil (SS) without dilution inoculation, used as negative control.

After microbiome manipulations, the soil moisture was maintained at 70% of the maximum water holding capacity (WHC), using deionized and sterilized water. This WHC was chosen to facilitate optimal conditions to promote microbial growth and re-establishment of the microbial community abundance. At that time, soil samples from all treatments were collected for chemical analysis, submitted to sequencing of bacterial and fungal ribosomal regions (V4 region of the 16S rDNA and ITS regions, respectively) and determination of acid phosphatase activity (AP_{ASE}) according to the method of Tabatabai (1994) and Tabatabai and Bremner (1969). For AP_{ASE} comparisons among soil treatment were performed by analysis of

variance (ANOVA, significance ≤ 0.05). Post hoc testing of significant results was performed using the LSD test ($p \leq 0.05$).

To characterise the soil, following parameters were determined on air dry soil (Raij et al. 2001): macronutrients (P, S, K, Ca, Mg); micronutrients (B, Cu, Fe, Mn, Zn); acidity features (pH_{CaCl2}, Al; H+Al) and organic matter (OM) (Table 1).

Table 1. Chemical analysis of soil samples, collected in the 0-20 cm layer in Piracicaba, Sao Paulo, Brazil, 20 days after manipulation of soil microbiome and before cultivation.

Treatment	pH	OM	P	S	K	Ca	Mg	Al	H+Al	B	Cu	Fe	Mn	Zn
	$CaCl_2$	g dm^{-3}	mg dm^{-3}		----------$mmol_c$ dm^{-3}----------					----------mg kg^{-1}----------				
NS	5.4	22	<3	18	<0.9	11	7	<2	15	<0.15	2.8	41	3.0	2.5
S50	5.0	22	4	8	<0.9	19	7	<2	15	<0.15	2.9	42	2.9	2.8
S80	5.0	23	6	<6	<0.9	19	9	<2	15	<0.15	3.0	40	3.0	2.5
S100	5.0	24	6	<6	<0.9	26	8	<2	13	<0.15	3.3	48	3.3	2.9
SS	4.8	25	4	11	<0.9	19	8	<2	15	0.19	2.1	53	16.0	2.2

Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS). Soil with dilution inoculation were considered similar to SS regarding chemical analysis. Chemical analysis abbreviations are defined as follows: OM: organic matter.

2.2.3 Total DNA extraction from soil and sequencing of partial bacterial 16S rDNA and fungal ITS

Total DNA was extracted from soil samples using a PowerSoil DNA isolation kit (MoBio, Carlsbad, EUA), according to the manufacturer instructions. From the total DNA extract, the V4 region of the bacterial 16S rDNA was amplified using 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3') primer combination (Caporaso et al. 2012). Similarly, the ITS region of fungi was amplified using ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') primer combination (Bokulich and Mills, 2013). These amplicons were prepared and sequenced for metabarcoding using a PGM Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA). Multiple samples were PCR-amplified using barcoded primers linked with the Ion adapter "A" sequence and Ion adapter "P1″ sequence. A two-base linker sequence was inserted between the adapter and the ITS primers to reduce any effect of the composite primer on PCR efficiency. PCR reactions were carried

out with the Platinum Taq DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA, USA). The PCR products were purified with Agencourt® AMPure® XP Reagent (Beckman Coulter, Brea, CA, USA), quantified by fluorometry using the Qubit Fluorometer kit-DNA High Sensitivity Assay kit (Invitrogen, Carlsbad, CA, USA) and combined in equimolar ratios. This composite sample was used for library preparation with the Ion OneTouch® 2 System fitted with the Ion PGM® OT2 400 Kit Template (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing was performed using the Ion PGM® Sequencing 400 kit on an Ion PGM® System, using the Ion 318® Chip v2.

A total of 364,394 16S and 1,185,242 ITS raw reads were processed using the Supercomputing Wales cluster. First, Ion torrent adapters were trimmed using cutadapt (Martin 2011) using a 10% mismatch threshold for removal. Trimmed sequences were then de-multiplexed, filtered, quality-checked, and clustered using a VSEARCH 2.3.2 (Rognes et al. 2016) pipeline modified from the Brazilian Microbiome Project (Pylro et al. 2014). All sequences with a maximum error value greater than 1 or shorter than 200 bp were removed for both 16S rDNA and ITS data. Operational Taxonomic Units (OTUs) were clustered using a closed-reference methodology at 97% similarity. Singletons were removed. Taxonomy was assigned to OTUs with QIIME 1.9.1 (Caporaso et al. 2010) with uclust methodology using the Greengenes 13.8 (DeSantis *et al*., 2006) and UNITE 8.0 (UNITE community, 2019) databases.

The subsequent OTU tables were processed using the phyloseq package (McMurdie and Holmes 2013) in R v. 3.5.1 (R Core Team, 2018). Both tables were filtered to remove non-target OTUs and rarefied 100 times, with the resulting mean richness being used for each sample. The final read depth for the bacterial OTU table was 929 and 1106 for the fungal OTU table.

For each dataset, mean OTU richness and Shannon-Weiner diversity (H') were calculated. Proportional abundances of bacteria and fungi at the class-level as well as fungal trophic groups were determined for each soil manipulation. The ITS OTU table was processed with FUNGuild (Nguyen et al. 2016) to determine fungal functional diversity. Subsequently, OTUs that matched to the FUNGuild database were categorized based on trophic mode to give an indication of the functional diversity resident in each soil treatment.

2.2.4 Pre-germination of plants and isolation of AMF spores

Brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*Crotalaria Juncea* L.) seeds were disinfected by immersing them in 2% (v/v) NaClO solution (15 min), and then

rinsing twice in deionized and sterile water. The seeds were sown in sterile and P-free quartz sand. After the germination, the seedlings were watered with sterile deionized water until being transplanted into the different soil treatments.

AMF species were selected based on the classification of mycorrhiza made by Chagnon et al. (2013) in groups: stress tolerant, ruderal, and competitor. Spores from AMF species were extracted from nursery pots of *Acalouspora colombiana* (Acalousporacea - stress tolerant); *Rhizophagus clarus* (Glomeracea - ruderal); and *Dentiscutata heterogama* (Gigasporacea - competitor). The inoculums belong to the collection of Department of Soil Science/Soil Microbiology Laboratory, "Luiz de Queiroz" College of Agriculture and Department of Natural Sciences, Regional University of Blumenau. Spore identification was based on information available on the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi website. All spores were immersed in 500mL sterilized deionized water and conditioned at 4 ºC until inoculation.

2.2.5 AMF inoculation of the plants, cultivation and harvest

The colonization experimental was set up 20 days after the manipulation of the soil microbiome. Seedlings (5 days old) were immersed in deionized and sterile water (30 min) to facilitate their removal from the sand. For brachiaria, three seedlings were transplanted into each pot, while for crotalaria one seedling was placed in each pot. At the point of transplantation, none of the roots showed any signs of mycorrhiza colonization, as determined by the blue method of Giovannetti and Mosse (1980) and Vierheilig et al. (1998). During transplantation, the roots were directly inoculated with 10 mL of water solution containing ~180 spores per pot. The pots were then placed in the glasshouse for 30 days until harvest. During this period, pots were irrigated with deionized and sterile water.

At harvest, plants were removed from the pots. Soil samples were kept refrigerated (4 ºC) for analysis of acid phosphatase activity. The increment or reduction in acid phosphatase activity ($AP_{ASE}d$) after cultivation with brachiaria or crotalaria ($AP_{ASE}f$) in relation to the AP_{ASE} before cultivation ($AP_{ASE}i$) was calculated based on the equation: $AP_{ASE}d = AP_{ASE}f - AP_{ASE}i$, where: $AP_{ASE}d$: variation in AP_{ASE} after cultivation in comparison to the AP_{ASE} before cultivation; $AP_{ASE}f$: AP_{ASE} after cultivation (brachiaria or crotalaria); $AP_{ASE}i$: AP_{ASE} before cultivation. Effects of soil modification on acid phosphatase activity, after cropping with brachiaria and crotalaria, were investigated using ANOVA (significance $p \leq 0.05$). Post Hoc testing of significant results was performed using the LSD test ($p \leq 0\ .05$).

The roots were washed from the soil under a steam of water and preserved in 70% alcohol prior mycorrhiza colonization analysis.

2.2.6 Determination of AMF colonization of roots

The ethanol preserved roots were washed under a steam of water and cut into 1 cm section, as described by Vierheilig et al. (1998). The excised roots were placed in 50 mL centrifuge tubes containing 10% (w/v) KOH and heated (90 ºC; 15 min) to remove the cytoplasmic contents and clarify the cortical tissue. Then the roots were washed with sterile tap water after which blue ink was added to stain the fungal structures contained within the roots. The samples were again heated (90 °C; 30 s) and stored in lactoglycerol solution (1:1:1; lactic acid, glycerol and water).

Mycorrhiza root colonization rates were quantified with the aid of a microscopy. For each root sample, three slides were made, containing ten ~1 cm root length segments, each slide. In total, 30 segments were analysed per sample. Rates were assigned according to the amount of fungal structures, as described by Trouvelot (1986).

Effects of soil modification on mycorrhiza colonization, after cropping with brachiaria and crotalaria, were investigated using ANOVA (significance $p \leq 0.05$). Post Hoc testing of significant results was performed using the LSD test ($p \leq 0.05$).

2.3 Results

2.3.1 Effect of soil manipulation on microbiomes

Soil microbiomes were changed by soil manipulations. For bacteria, the highest richness and H' were observed under NS, S50, S80 and SS (Table 2). For fungi, the highest number of observed OTU richness and diversity were detected under S50, followed by NS and S80. On the other hand, low richness and H', for both bacteria and fungi, were observed under the heat shock treatment S100 and dilutions $SS+10^{-1}$; $SS+10^{-3}$ and $SS+10^{-6}$ (Table 2).

Table 2. OTU richness and Shannon (H') index of a soil sample collected in the 0-20 cm layer in Piracicaba, Sao Paulo, Brazil, 20 days after manipulation of soil microbiome and before cultivation.

Treatment	OTU richness		Shannon (H')	
	Bacteria	Fungi	Bacteria	Fungi
NS	187.8±8.5	84.2±24.7	4.69±0.09	2.40±1.22
S50	186.4±9.2	102.8±17.4	4.58±0.06	3.28±0.83
S80	188.9±12.5	76.5±2.3	4.54±0.18	2.38±0.24
S100	52.0±4.1	51.4±0.6	2.72±0.20	2.70±0.34
$SS+10^{-1}$	109.7±13.3	36.3±8.1	3.70±0.18	1.98±0.17
$SS+10^{-3}$	84.0±17.4	40.7±4.9	3.47±0.23	2.43±0.33
$SS+10^{-6}$	88.0±21.1	36.0±0.2	3.34±0.17	2.05±0.11
SS	179.7±4.4	70.1±6.1	4.44±0.06	2.29±0.33

Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$); and without dilution inoculation (SS).

The S50 and S80 treatments showed high similarity in comparison to NS based on bacterial metabarcoding at the class-level (Figure 1A). Bacterial communities in soils from the S100 treatment demonstrated a major shift in proportional abundance. There was a marked increase in proportion of Betaproteobacteria and a loss of minor constituents of the NS community. Additionally, Sphingobacteriia and Bacilli became prominent under S100 treatment. Regarding to the dilution extinction method, compared to the NS, the most abundant class were favoured, and a reduction in abundance in the less abundant in NS was noticed in $SS+10^{-1}$, $SS+10^{-3}$ and $SS+10^{-6}$. In contrast, the classes Sphingobacteria and Bacilli were observed only in the dilution treatments. The SS treatment, without dilution inoculation, was very similar to NS for most of the classes, except for the presence of Bacilli and loss of Gammaproteobacteria (Figure 1A).

For fungi, Leotiomycetes showed high abundance in NS, but this was reduced in the S50 and S80 treatments; this class was not observed at significant levels in the S100, $SS+10^{-1}$, $SS+10^{-3}$, and $SS+10^{-6}$ treatments. In SS, this class was detected, but in lower abundance compared to the NS. On the other hand, Sordariomycetes were favoured by all the treatment compared to the NS, increasing the abundance, except for SS (Figure 1B).

Regarding fungal functional groups, representatives from the most important classifications, namely, symbiotrophs (SY), saprotrophs (SA), and pathotrophs (PA) were maintained in most treatments, as were organisms that may fit into (Figure 1C). Considering the heat shock method, increasing temperature generated a reduction gradient in abundance of organisms classified as PA, PA-SA, and SA-SY, and very low abundance or absence of these groups were observed under S100. In contrast, the abundance of SA organisms increased for all the temperatures treatments, compared to NS, while SY organisms increased according to the temperatures used in the heat shock method, where the highest abundance of SY was observed in S100. Organisms classified as SA-SY were observed only under S80 and S100 (Figure 1C).

For the dilution extinction method, the PA-SY was observed in very diluted treatment, and a lower increment in SA was observed for the dilutions. Under SS treatment, high abundance of PA and SA-SY was observed, but these groups were not observed in the dilution inoculation treatments (Figure 1C).

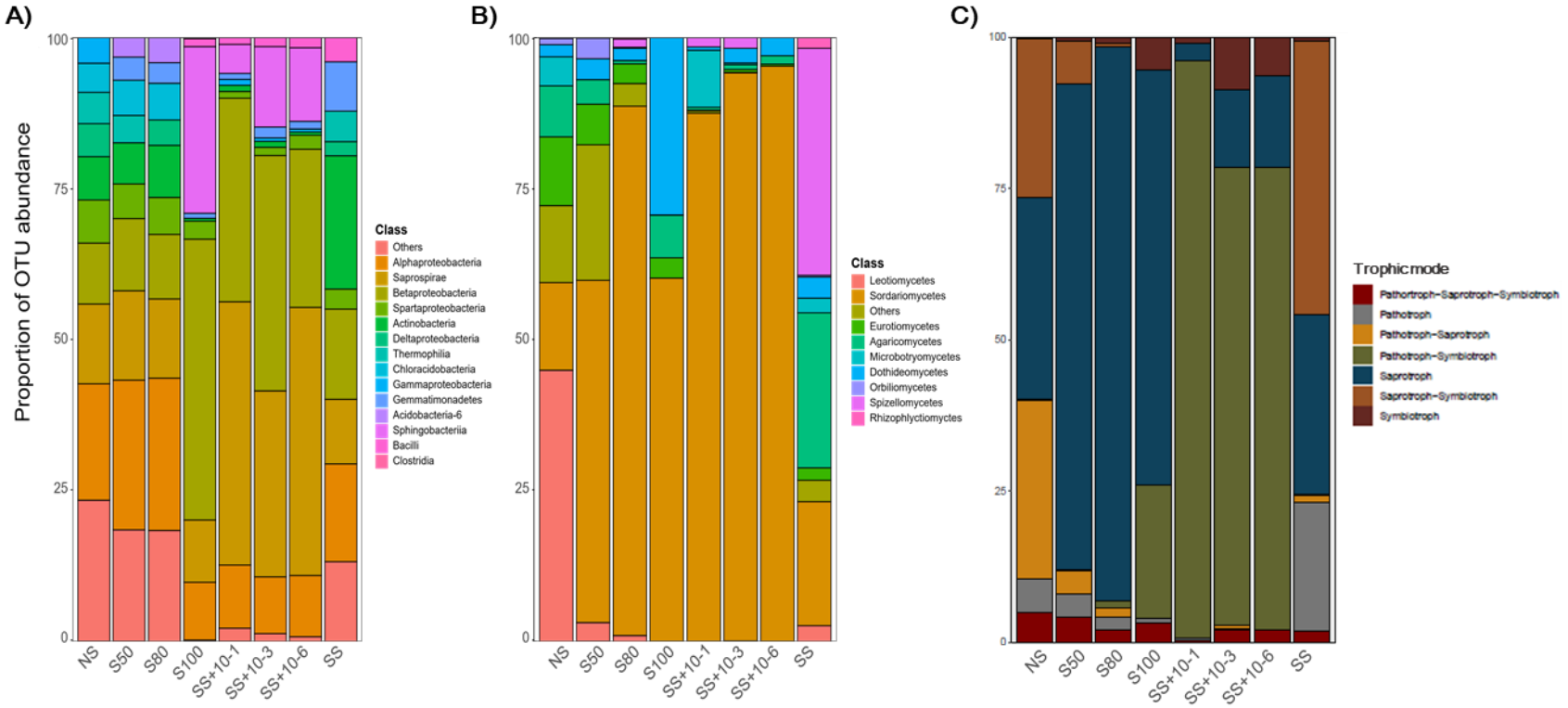

Figure 1. Frequency of classes for bacteria (**A**) and fungi (**B**), and the functional assignment of fungi by Funguild (**C**) from soils sampled 20 days after soil microbiome manipulation. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$); and without dilution inoculation (SS).

2.3.2 Effect of soil manipulation in the acid phosphatase activity

No differences between NS, S50 and S80 were observed ($p \leq 0.05$) regarding acid phosphatase activity. However, in the S100 treatment acid phosphatase activity reduced ($p \leq 0.05$). In the microbiome diluted treatments, a significant ($p \leq 0.05$) trend was observed, with lower values of enzyme activity in more diluted treatments (Figure 2).

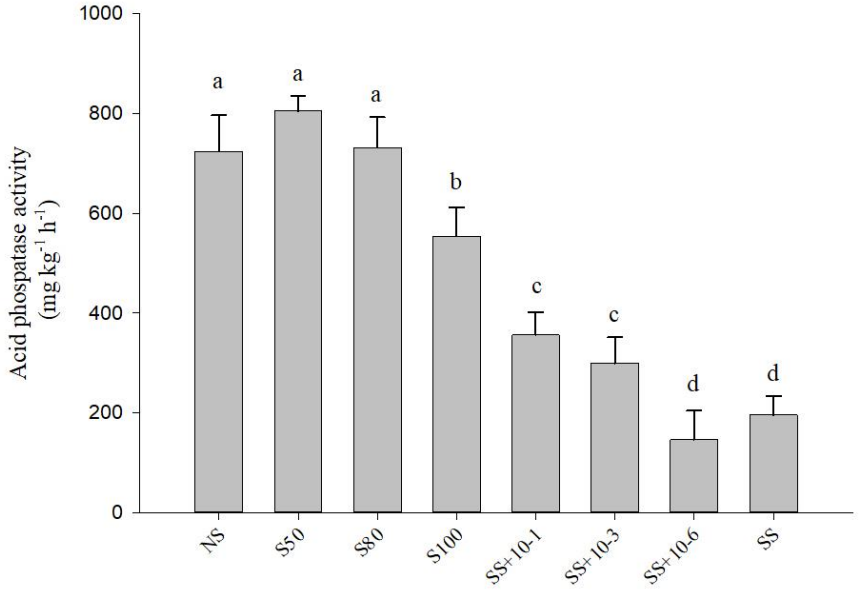

Figure 2. Acid phosphatase activity 20 days after soil microbiome manipulation. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$); and without dilution inoculation (SS). Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test.

2.3.3 Dependency of brachiaria and crotalaria on microbial community for mycorrhiza colonization

Brachiaria showed distinctions in mycorrhiza colonization rate according to microbiome manipulation. NS showed the highest colonization in the absence of AMF spore inoculation ($p \leq 0.05$) (Figure 3A), with 19% of colonization. On the other hand, when AMF spores were inoculated, either for *A. colombiana, R. clarus* or *D. heterogama*, the rate of colonization increased under NS treatment (54%, 38%, and 43%, respectively). S50 showed AMF colonization similar to NS following inoculation of all AMF species. A reduction compared to

NS and no differences were observed for AMF colonization according to microbiome manipulation treatments, with or without inoculation (Figure 3).

In contrast, crotalaria did not show the same tendency for changes in the mycorrhiza colonization regarding the manipulated soil microbiomes (Figure 4). When AMF was not inoculated, there was an increase ($p \leq 0.05$) in the rates of mycorrhiza colonization in S80, followed by NS, S50, and no difference ($p > 0.05$) under S100, and SS, even when dilutions were inoculated, were observed (Figure 4A). When *A. colombiana* was inoculated, the highest ($p \leq 0.05$) colonization was observed in the S50, following by SS+10^{-1}; SS+10^{-6}, and SS without difference ($p > 0.05$) observed. Under *Acalouspora colombiana* inoculation the lowest ($p \leq 0.05$) colonization rate was observed in S100 soils (Figure 4B). When *R. clarus* was inoculated, the highest ($p \leq 0.05$) colonization was observed in the S80 soils, followed by the NS. Other treatments did not differ to each other ($p > 0.05$) (Figure 4C). The results for the inoculation of *D. heterogama* revealed the highest ($p \leq 0.05$) colonization in the treatment SS+10^{-6}, statistically similar to treatments NS and SS+10^{-1}. Intermediate values were observed in S80 and SS and the lowest ($p \leq 0.05$) colonization was observed in the S50 and S100 (Figure 4D).

However, it is worth mentioning that the inoculation of specific AMF species was effective to promote colonization into the roots of crotalaria, regardless the level of microbial diversity. Under NS, S50, and S80, for all the AMF species that were inoculated, and no AMF inoculation, rates of mycorrhiza colonization were observed (Figure 5A, B, C). However, when crotalaria was cultivated in the treatment S100 no colonization was detected for any mycorrhiza species inoculated ($p > 0.05$) (Figure 5D). Soil sterilization, either without or with inoculation of dilution, did not inhibit the rates of colonization when *A. colombiana* and *D. heterogama*, but when either no AMF or *R. clarus* was inoculated, the colonization of crotalaria with mycorrhiza was not observed (Figure 5E, F, G).

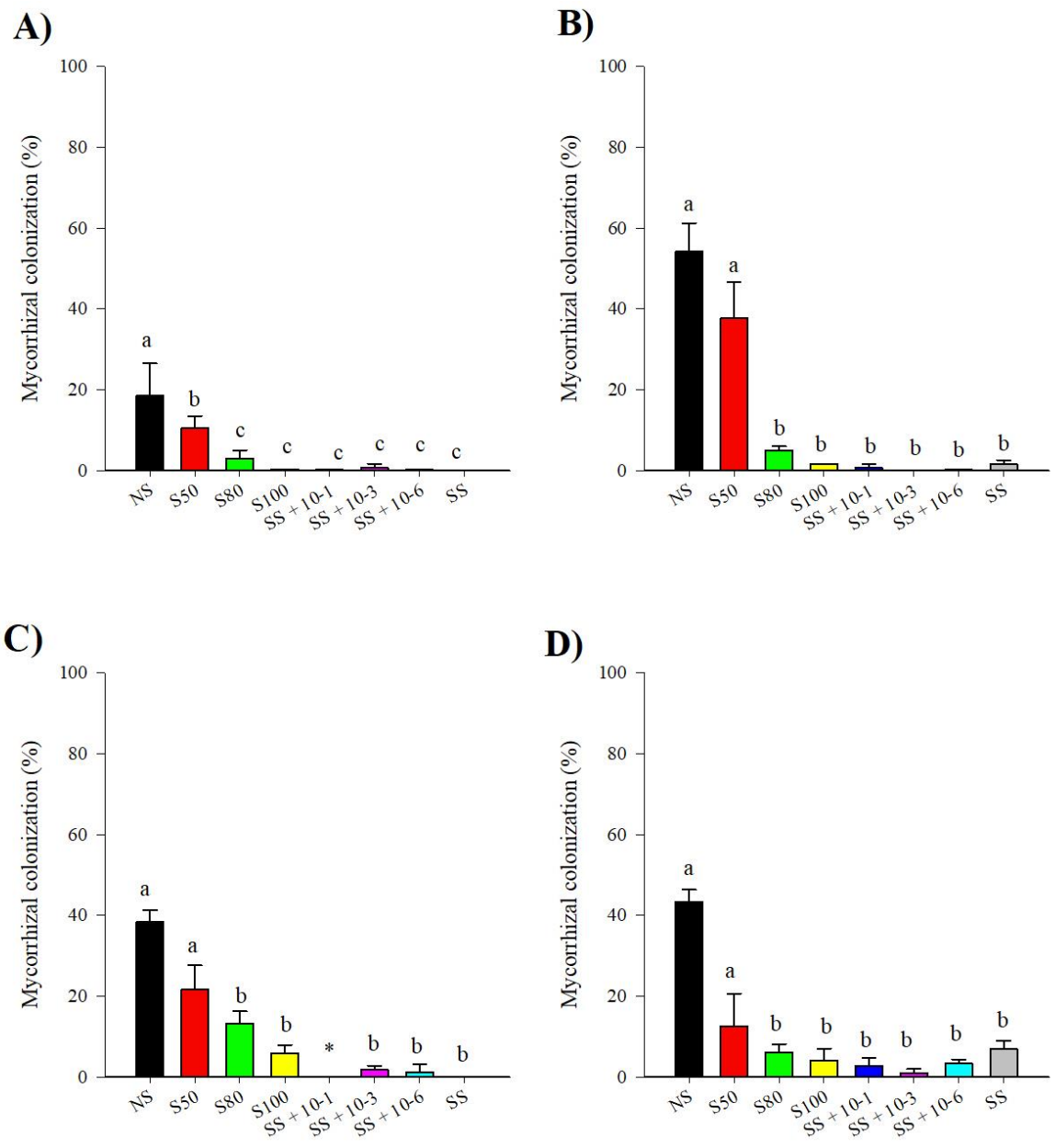

Figure 3. Mycorrhiza colonization of brachiaria cultivated in a soil submitted to distinct manipulated microbiome. Plants were inoculated with: No AMF (**A**); *Acalouspora colombiana* (**B**); *Rhizophagus clarus* **(C**); and *Dentiscutata heterogama* **(D**). Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$); and without dilution inoculation. Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test. *missed data

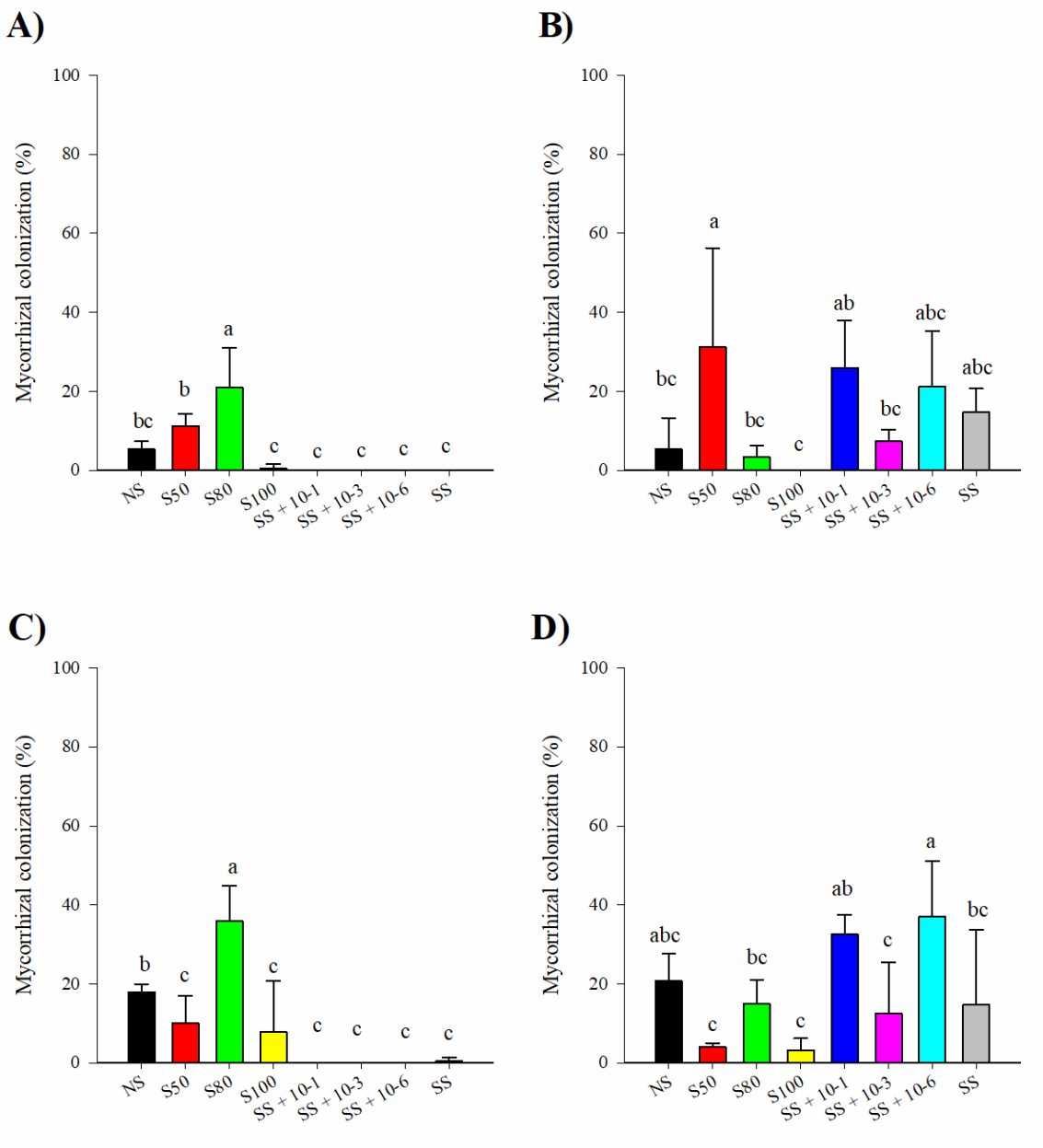

Figure 4. Mycorrhiza colonization of crotalaria cultivated in a soil submitted to distinct manipulated microbiome. Plants were inoculated with: No AMF (**A**); *Acalouspora colombiana* (**B**); *Rhizophagus clarus* **(C**); and *Dentiscutata heterogama* **(D**). Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$); and without dilution inoculation. Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test.

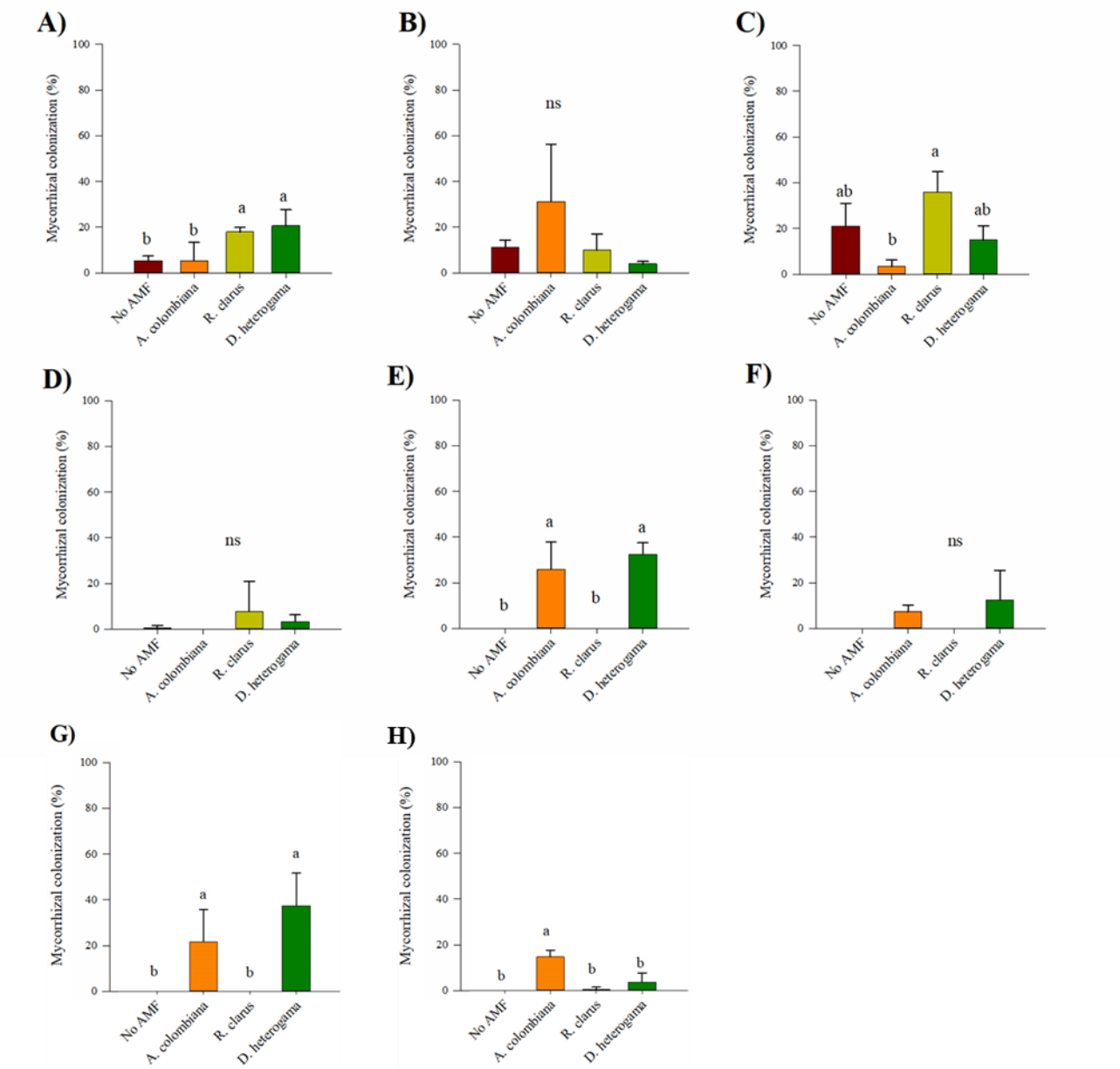

Figure 5. Mycorrhiza colonization of crotalaria cultivated without or with inoculation of mycorrhiza arbuscular fungi spores in a soil submitted to natural soil (**A**); soil submitted to 50 ºC for one hour (**B**), soil submitted to 80 ºC for one hour **(C**); soil submitted to 100 ºC for one hour (**D**); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) at ($SS+10^{-1}$) **(E)**; ($SS+10^{-3}$) **(F)**; ($SS+10^{-6}$) **(G)**; and no inoculation (SS) **(H)**. Different letters show significant difference between arbuscular mycorrhiza specie treatments ($p \leq 0.05$) by LSD test

2.3.4 Acid phosphatase activity and mycorrhiza colonization

Mycorrhiza colonization and the difference in AP_{ASE} promoted by brachiaria, in comparison to the initial level, regardless the AMF inoculation, showed a negative correlation. High levels of mycorrhiza colonization and low modifications or even a reduction in AP_{ASE} activity were observed in NS and S50 (Figure 6). For S80 and S100 soils, low mycorrhiza colonization and low modifications were observed compared to the initial levels of AP_{ASE}. However, when the dilution was used, low mycorrhiza colonization was observed, with an increment in AP_{ASE} (Figure 6). For crotalaria, mycorrhiza colonization and AP_{ASE} did not show any correlation according to the microbiome manipulation and AMF inoculated (Figure 7).

Although a difference in AP_{ASE} was observed after isolation of the effect plant, the trend across the microbial manipulation for AP_{ASE} was maintained. In general, high AP_{ASE} was observed under NS, intermediary in a gradient for the manipulation and the lowest values under SS (Figure 8 and 9). This indicates that the changes promoted by the plants, mainly by brachiaria, concern to the reduction under NS and the increment under SS, were not sufficient to equalize the initial levels for microbial manipulation after crop.

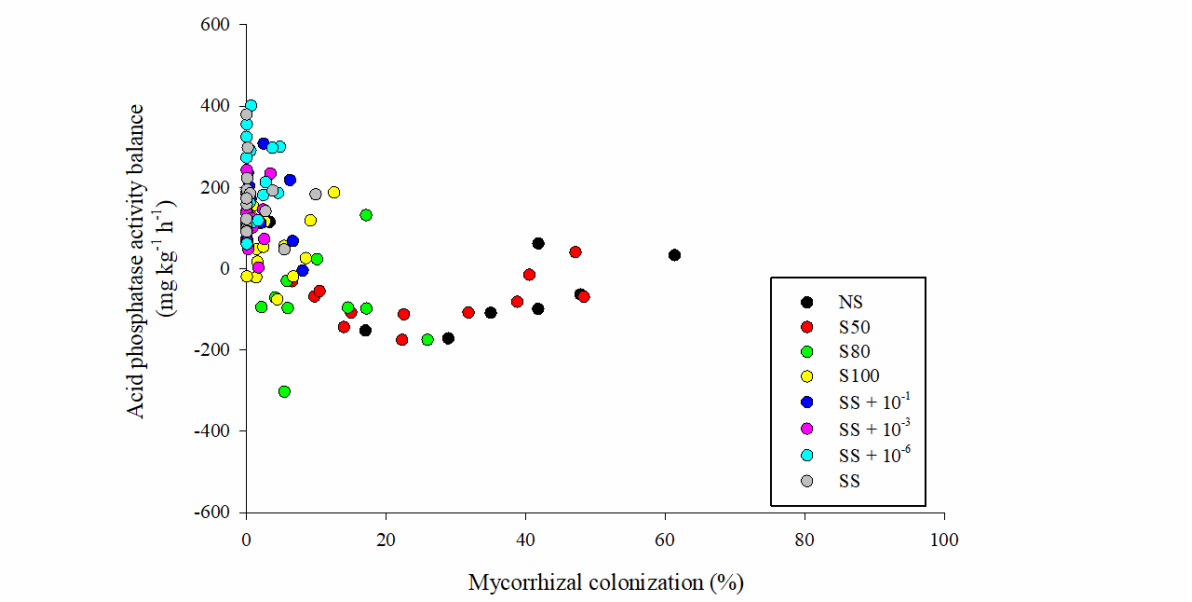

Figure 6. Correlation between the acid phosphatase activity balance and mycorrhiza colonization after brachiaria cultivation in soils subjected to distinct microbiome manipulations. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$). Plants were inoculated with: No AMF; *Acalouspora colombiana*; *Rhizophagus clarus*; *Dentiscutata heterogama.* Values above 0 in the axis y mean increase in AP_{ASE} in relation to the soil before cultivation. Values bellow 0 in the axis y mean decrease in AP_{ASE} in relation to the soil before cultivation.

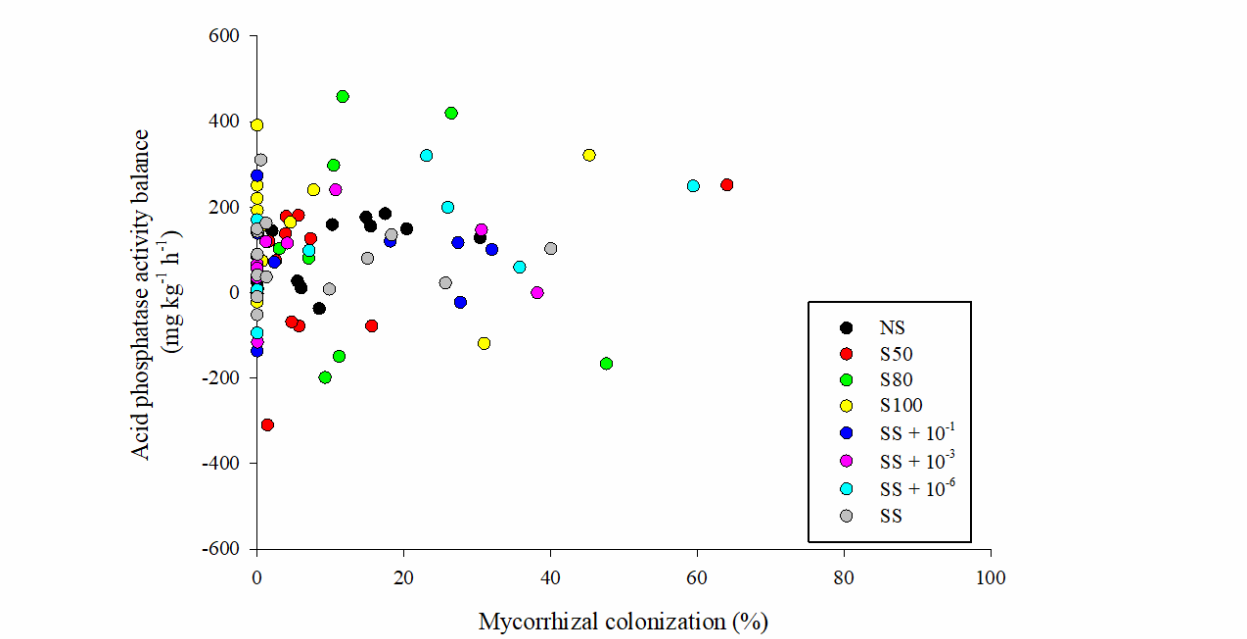

Figure 7. Correlation between the acid phosphatase activity balance and mycorrhiza colonization after crotalaria cultivation in soils subjected to distinct microbiome manipulations. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$). Plants were inoculated with: No AMF; *Acalouspora colombiana*; *Rhizophagus clarus*; *Dentiscutata heterogama.* Values above 0 in the axis y mean increase in AP_{ASE} in relation to the soil before cultivation. Values bellow 0 in the axis y mean decrease in AP_{ASE} in relation to the soil before cultivation.

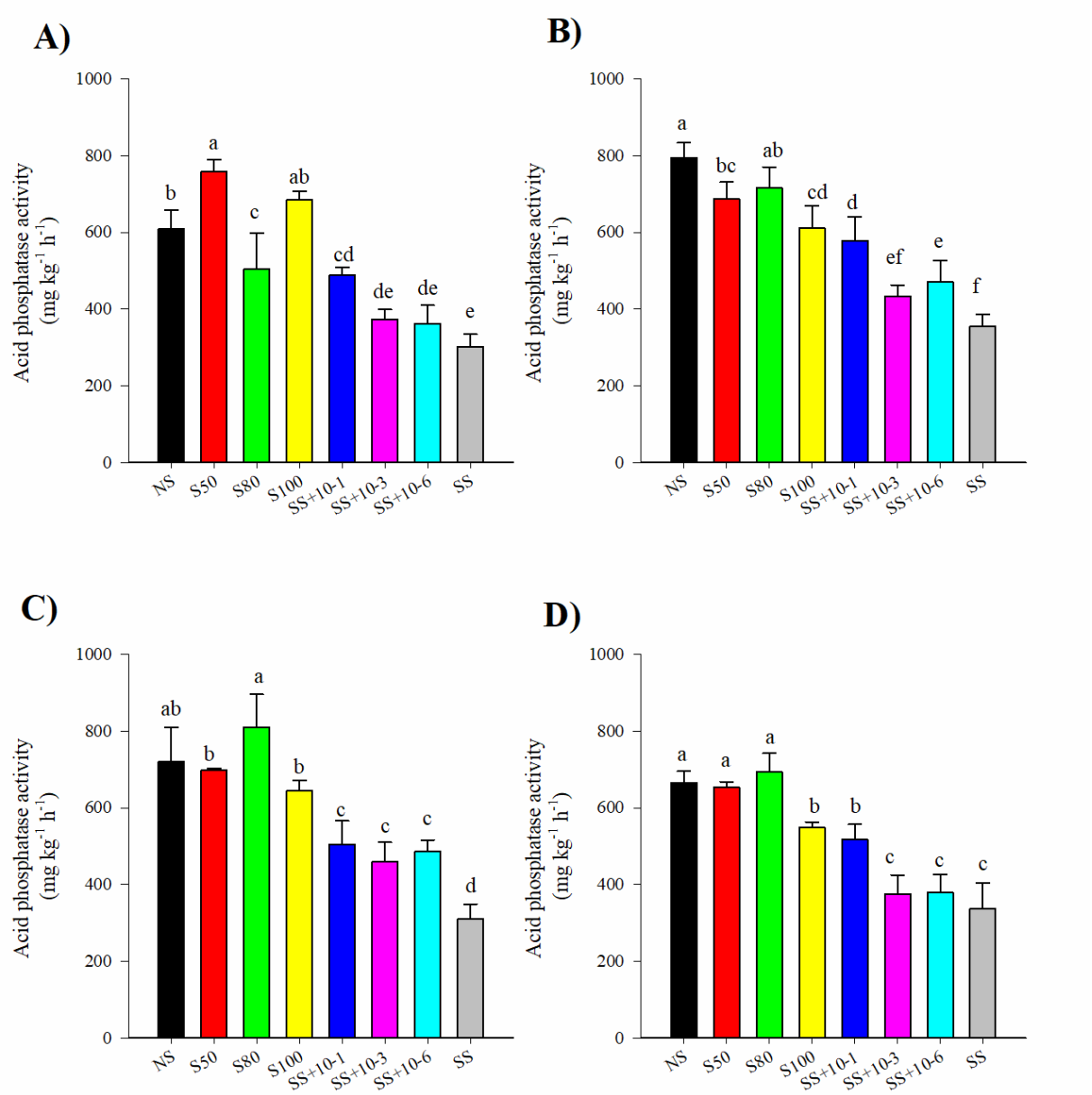

Figure 8. Acid phosphatase activity of brachiaria cultivated in a soil submitted to distinct manipulated microbiome. Plants were inoculated with: No AMF **(A)**; *Acalouspora colombiana* (**B**); *Rhizophagus clarus* **(C)**; and *Dentiscutata heterogama* **(D)**. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$); and without dilution inoculation. Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test.

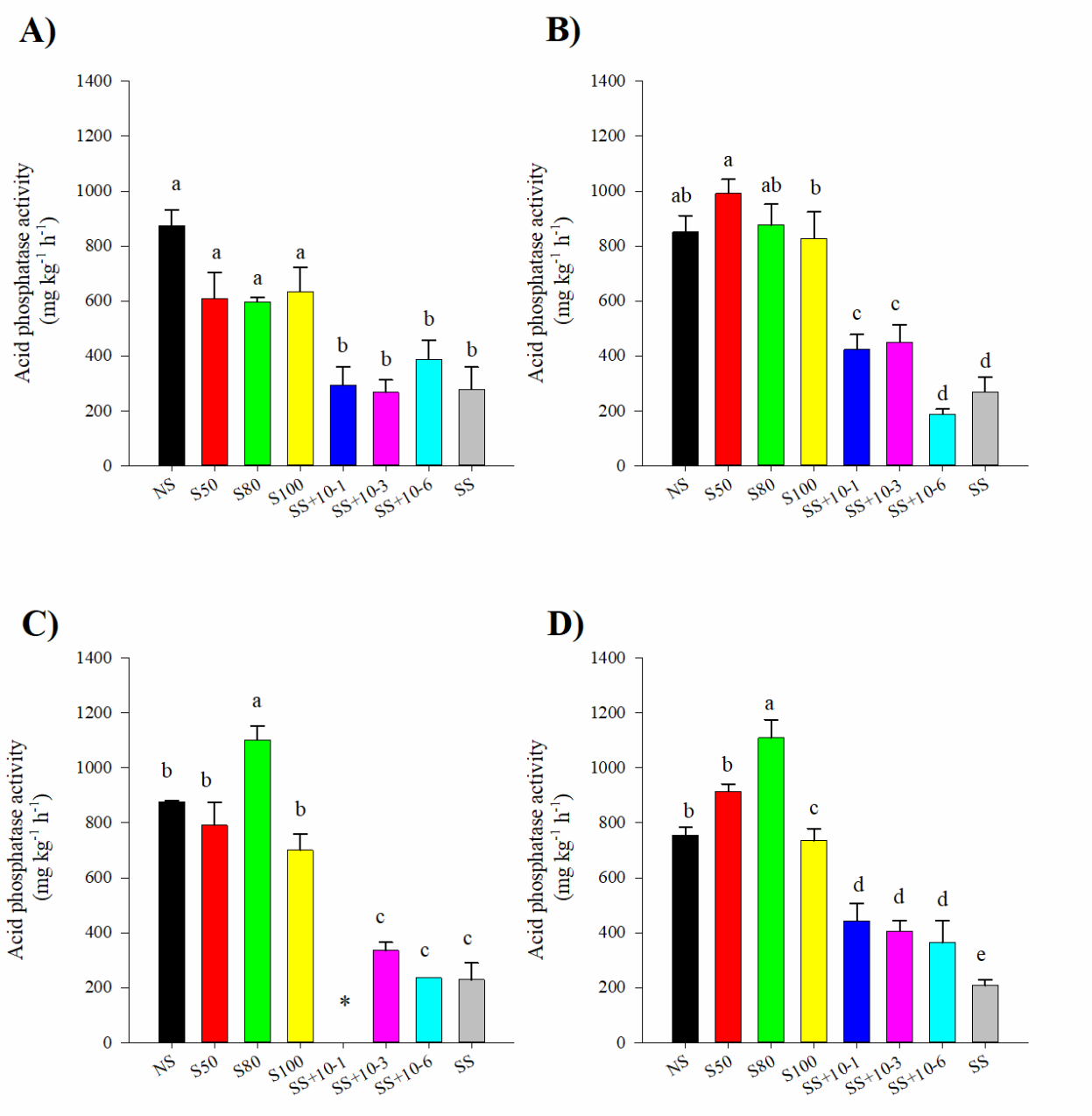

Figure 9. Acid phosphatase activity of crotalaria cultivated in a soil submitted to distinct manipulated microbiome. Plants were inoculated with: No AMF **(A)**; *Acalouspora colombiana* (**B**); *Rhizophagus clarus* **(C)**; and *Dentiscutata heterogama* **(D)**. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-1}$); ($SS+10^{-3}$); ($SS+10^{-6}$); and without dilution inoculation. Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test. *missed data.

2.4 Discussion

Connections between soil microbiome diversity and plants acting through synergistic and symbiotic associations, affecting the whole ecosystem processes, such as, energy and nutrients flow (Beinart 2019). Although arbuscular mycorrhiza fungi (AMF) are known to interact with plant, its connections established with other components of the soil microbiome are still to be described, based on evidences of specific groups of organisms that may help in the associations between plant and AMF (Artursson et al. 2006). However, the role of the soil microbiome diversity and composition upon this process is still to be explored.

We used two methods to promote the microbial extinction in soil samples, the heat shock and the dilution of the native community. By using the heat shock method, the resistance to temperature was expected to determine the new community (Nottingham et al. 2016). On the other hand, in the dilution approach, we expected to change the community based on the abundance of populations in the natural soil, once the strength of any dilution effect also depends on the relative abundance of the dilution (Johnson and Thieltges 2010).

The manipulation of the soil microbiomes resulted in distinct changes from natural communities for both bacteria and fungi, with evidence of differential selection processes between the methods used. By using heat shock the changes in the bacterial community were most observed under S100, with loss of some taxa, indicating that the thermoresistance of the microbiome in the NS was limited to 50 °C and 80 °C, that showed similar microbial community to NS. Allison and Martiny (2008) in a review observed that, even if microorganisms are sensitive to a disturbance, the community might still be resilient and quickly return to its composition before the disturbance.

The treatment with more similarity to the NS, based in richness and diversity for both bacteria and fungi, was the sterilized soil (SS). This result is probable due to a large portion of the DNA detected from those samples as relic DNA (Carini et al. 2017). When dilutions were inoculated, the relic DNA signal was lost by biomass incorporation through the recolonization of the soil by the species added by inoculation, that compose the new manipulated communities.

Although the non-inoculated autoclaved soil showed high similarity with natural soil, the enzymatic activity (AP_{ASE}) was the lowest in this system. It reinforces that the DNA found in the sequencing was not from active organisms. Also, the increment in temperature of the soil treatments gradually reduced the enzymatic activity. Serrasolsas and Khanna (1995) analysed the

changes promoted by heating and autoclaving in forest soils, concluded that the reduction in the activity of phosphatase activity according to the increment of temperature is due to death of the microorganisms and denatured enzymes. However, our results showed that the inoculation of dilution in the autoclaved soil reduced the negative impact in the enzymatic activity. The soil enzymes can be found free in the soil, immobilized by the microorganisms or adsorbed in the soil particles. Kandeler (1990) characterized phosphatases activity free and adsorbed in soils, and observed that the suspension showed high activity in the soil compared to the enzyme extracted. Thus, the sequential dilution may reflect in the inoculation of free enzymes in the soil, in gradients according to the level of dilution.

Once we obtained distinct soil microbiomes by using different approaches, we tested the influenced of the new assembled communities upon plant-AMF association.

For brachiaria the response was maintained, for all the three AMF species evaluated regardless soil manipulation methods, and high mycorrhizal colonization in the natural soil, possibly indicating a clear dependency on soil microbial diversity and richness to properly assembly the roots - AMF functional system.

Yet, crotalaria revealed a more specific response for AMF colonization, depending on method used and AMF inoculated. Under higher diversity, all the AMF species inoculated showed expressive colonization, but *A. colombiana* and *D. heterogama* showed high colonization, even in very disturbed system, such as autoclaved soil, without dilution inoculation, therefore, crotalaria showed less dependency in the microbiome. Thus, the absence of the microorganisms lost, and the condition created in the autoclaved soil, either without or with inoculation, did not affect the colonization for crotalaria by these two AMF species.

Based on the classification of AMF in groups made by Chagnon et al. (2013) *A. colombiana* is a stress tolerant specie and *D. heterogama* was classified as a competitor. On the other hand, *R. clarus*, that did not colonize crotalaria under the same condition, was classified as a ruderal. According to this classification, when AM fungi is submitted to a stressed condition, such as the autoclaving procedure used here, the turnover rates across a broader phylogenetic spectrum may reveal that some taxa, classified as stress tolerant, use plant carbon more efficiently, compared to a ruderal. Furthermore, the competitive AM fungi should be those with functional traits that improve carbon acquisition from the host plant, which may drive the affinity of crotalaria for *D. heterogama*.

Therefore, the presence of specific groups of organisms, that were supposed to benefit the associations with AMF, may not guarantee the colonization of the roots, depending on the plant in question. Kurth et al. (2013) detected and quantified the MHB and a mycorrhizal fungus in plant-soil microcosms, at different levels of complexity, and concluded that not only the presence of microorganisms but plant roots can affect the nature of MHB-fungus interactions. This supports our finding that despite being cultivated in soil submitted to the same microbial communities, brachiaria and crotalaria did not show the same response for mycorrhizal colonization, indicating that the MHB for one crop may not be efficient for another. Likewise, Chagnon et al. (2019) analysed the mycorrhizal network assembly using 20 plant species and concluded that plants were not equally affected by the presence of neighbours.

Another point of consideration for improving the plant P uptake is the acid phosphatase, that may be originated by plants and microorganisms, mediating phosphorus availability by mineralization of the organic phosphate (Richardson and Simpson 2011). In this sense, a study compilation of many works relating mycorrhiza to the AP_{ASE} are present in a review made by Joner et al. (2000). Our results showed that the trade-off observed between AMF colonization and AP_{ASE} was also differential between brachiaria and crotalaria. Whereas for brachiaria we found a clear trend, showing a possible substitution pattern between these processes, for crotalaria this trend was absent. For brachiaria, in systems with more functional diversity, high colonization was related to no much changes, and even a reduction in AP_{ASE} was observed, indicating that the association with mycorrhiza was dominant for brachiaria, rather than AP_{ASE} exudation. Azcon et al. (1982) observed that mycorrhizal colonization decreased the phosphatase activity of lavender (*Lavandula spica* Cav.) root surface compared with non-mycorrhizal control plants, indicating that the mycorrhiza not always is related to increments in AP_{ASE}. Farther, Dodd et al. (1987) observed that plants colonized by *Glomus monosporum* did not increase the AP_{ASE} activity associated with roots, but increased plant dry matter, which was related to the high efficiency of this fungus isolate to promote plant P uptake. Our results showed that under S100 and the serial dilution method, low colonization and high acid phosphates activity of brachiaria were observed, which may be due to the low functional diversity, which encourage the plant to produce acid phosphatase itself, or the microorganisms around, to access the P from the soil. Therefore, when the association between the mycorrhiza and the plant is not successful, plants may spend energy to produce or to stimulate the production of enzyme to uptake P.

Conversely, crotalaria showed independence for microbial diversity to promote the root colonization with mycorrhiza and acid phosphatase activity. Joner and Jakobsen (1995) studied the extracellular phosphatase activity of arbuscular mycorrhizal hyphae and concluded that the influence of AM hyphae on phosphatase activity in root-free soil seem to be indirect through interactions with other microorganisms. Thus, the lack of correlation between the AP_{ASE} and the colonization may indicate that the enzymatic balance detected in soil cultivated with crotalaria here was more related to the interaction between the microbial community and AMF specie, showing different response according to the microbiome around.

2.5 Conclusion summary

Our results first evidenced that the methods of microbial manipulation tested were able to create different microbial communities.

In addition, the mycorrhizal colonization and acid phosphatase activity were related to the structure of the soil microbiome, depending on the crop analysed. Brachiaria showed a dependency on the microbial community to be colonized and presented a compensation between the mycorrhization and $AP_{ASE.}$ Crotalaria, showed that the inoculation of specific AMF species was more relevant for mycorrhiza colonization, regardless, the microbiome present in the disturbed soil system.

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3 CORRELATION BETWEEN MICROSCOPE ANALYSIS AND REAL TIME PCR FOR MYCORRHIZA COLONIZATION FROM ROOT SAMPLES

Abstract
Due to the low standardization for mycorrhiza quantification by using the microscope approaches, the objective of this study was to test a qPCR technique to quantify the mycorrhiza colonization in root samples. The experiment was conducted in a glasshouse, using soils treated with eight distinct compositions of the native microbiome: natural soil (i); soil exposed to heat treatments for one hour of 50 ºC (ii), 80 ºC (iii) or 100 ºC (iv); and soils autoclaved twice (121 ºC, 103 kPa, 1 h) to generate the sterilized soil (SS) followed by inoculation of dilutions (10% v/w of natural soil): $SS+10^{-1}$ (v); $SS+10^{-3}$ (vi); $SS+10^{-6}$ (vii); SS (without dilution inoculation) (viii). Seedlings of brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*Crotalaria juncea* L.) were transplanted to soils with separately inoculations of three AMF species (*Acalouspora colombiana*, *Rhizophagus clarus* or *Dentiscutata heterogama*). At the harvest, half of the roots were sampled for mycorrhizal quantification by using microscope (kept in 70% ethanol) and half was sampled for molecular analysis of mycorrhizal colonization (kept in -20°C). The samples kept in ethanol were dyed and analysed in microscope according to rates of colonization, in percentage. Samples in a range of 0% and the maximum colonization were selected (26 samples for brachiaria and 24 samples for crotalaria). From the other halves of the selected samples, kept in -20°C, the DNA was extracted, and qPCR using the primers FLR3 and FLR4, for mycorriza, was performed. A correlation between the percentage of colonization obtained by microscope and the qPCR was made. Our results showed that the set of primer FLR3 and FLR4 was promising to correlate qPCR and microscope analysis, showing better correlation for brachiaria compared to crotalaria, and under inoculation of *A. colombiana* and *D. heterogama* compared to *R. clarus*.

Key words: Molecular approach; *Urochloa brizantha* cv. Marandu; *Crotalaria juncea* L.

3.1 Introduction

Quantification of mycorrhizal colonization rates is traditionally done by observing and accounting for the presence and absence of AMF structures in root fragments. For this, the root samples are collected and stored in 70% alcohol solution, and to prepare for analysis the roots are washed with water to remove excess alcohol, bleached with 10% KOH solution and stained with blue ink (Vierheilig et al. 1998). Then, the roots are dispose in slides and the colonization is graded according to Trouvelot (1986), in microscope; or the roots are dispersed in petri dishes with a checkered bottom and observed, with the aid of a stereomicroscope, to account for the relative presence of histological AMF materials based on the frequency of observation, following a gridline methodology (Giovannetti and Mosse 1980). Possession of the counting data can

consider: i) the most likely number of infectious purposes; ii) infection units; iii) mean percentage of infection.

Once this analysis is subjective because requires knowledge and experience, mistakes in the interpretation may lead to wrong conclusions regarding to the mycorrhiza colonization (Brundrett and Tedersoo 2019).

Therefore, a standardization in the microscope analysis of mycorrhiza is required. In this sense, Stoian et al. (2019) developed a new technic based on the digitalization of the microscopic observations of colonization and a methodological framework for obtaining objective and comparable microscopic observations.

Another approach that has being used is the molecular methodologies to determine by using specific primers for mycorrhization, but the application of this technique dependent on the specificity and optimization of the use of primers. Some author developed specific primers for Glomeromycota phylum (Gollotte et al. 2004; Krüger et al. 2009), that may be used for this propose.

In recent years, due to the advancement of quantitative molecular techniques, such as the real time polymerase chain reaction (qPCR), it is possible to quantify the number of target genes or functional genes per gram of samples produced in each PCR cycle, and so be used for mycorrhizal quantification (Voříšková et al. 2017).

The hypothesis of this study was that qPCR may reduce the subjectivity of the quantification of mycorrhiza.

The objective of this study was to test the qPCR to complement the microscope technique for quantification of mycorrhizal colonization rates in roots samples.

3.2 Material and methods

3.2.1 Experimental setup in glasshouse

The experiment was conducted in a glasshouse (27°C ± 3), in a randomized block design, with eight treatments and four replicates. Each experimental unit comprised a plastic pot (5 cm internal diameter × 9 cm tall), containing 270g of soil (dry weight). Soil was classified as a sandy clay loam textured Ferralsol (WRB-FAO, 2015), collected from a depth of 0-20 cm within a

livestock-grazed pasture, in Piracicaba, Sao Paulo, Brazil (22°43'03.0''S, 47°37'00.4''W). The main characteristics of this soil is presented in table 1, as natural soil (NS).

To generate gradient of root colonization, the soil was treated to generate eight different condition: i) natural soil (NS); three separate microbiome treatments were generated by the heat the soil as follows: ii) 50 ºC for 1 h (S50), iii) 80 ºC for 1 h (S80); iv) 100 ºC for 1 h (S100), and four treatments were obtained by first sterilizing the soil by autoclaving (SS) twice (121 ºC, 103 kPa, 1 h) (Table 1). After 7 days, soils aliquots were re-inoculated using a dilution-to-extinction method (van Elsas et al. 2012). This approach generated the treatments v) SS+10^{-1} (10% v/w of natural soil dilution added to the sterile soil); vi) SS+10^{-3}; vii) SS+10^{-6}; and viii) SS, without dilution inoculation, used as negative control (table 1).

Table 1. Chemical analysis of a soil sample, collected in the 0-20 cm layer in Piracicaba, Sao Paulo, Brazil, 20 days after manipulation of soil microbiome and before cultivation.

Treatment	pH	OM	P	S	K	Ca	Mg	Al	H+Al	B	Cu	Fe	Mn	Zn
	$CaCl_2$	g dm^{-3}	mg dm^{-3}		----------mmol$_c$ dm^{-3}----------					----------mg kg^{-1}----------				
NS	5.4	22	<3	18	<0.9	11	7	<2	15	<0.15	2.8	41	3.0	2.5
S50	5.0	22	4	8	<0.9	19	7	<2	15	<0.15	2.9	42	2.9	2.8
S80	5.0	23	6	<6	<0.9	19	9	<2	15	<0.15	3.0	40	3.0	2.5
S100	5.0	24	6	<6	<0.9	26	8	<2	13	<0.15	3.3	48	3.3	2.9
SS	4.8	25	4	11	<0.9	19	8	<2	15	0.19	2.1	53	16.0	2.2

Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS). Soil with dilution inoculation were considered similar to SS regarding chemical analysis. Chemical analysis abbreviations are defined as follows: OM: organic matter.

Brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*Crotalaria juncea* L.) seeds were disinfected by immersing them in 2% (v/v) NaClO solution (15 min), and then rinsing twice in sterile deionized water. The seeds were sown in sterile and P-free quartz sand.

Spores from AMF species were extracted from nursery pots of *Acalouspora colombiana* (Acalousporacea); *Rhizophagus clarus* (Glomeracea); and *Dentiscutata heterogama* (Gigasporacea).

3.2.2 AMF inoculation of the plants and cultivation

The colonization experimental was set up 20 days after the manipulation of the soil microbiome. Seedlings (5 days old) were immersed in sterile deionized water (30 min) to facilitate their removal from the sand. For brachiaria, three seedlings were transplanted into each pot, while for crotalaria one seedling was placed in each pot. During transplantation, the roots were directly inoculated with 10 mL of water solution containing ~180 spores per pot. The pots were then placed in the glasshouse for 30 days until harvest. During this period, pots were irrigated with deionized and sterile water.

3.2.3 Root sampling

At harvest, plants were removed from the pots. The roots were washed from the soil under a steam of water. Part of the roots was preserved in 70% alcohol prior mycorrhiza colonization analysis by microscope and part of the roots was keep in -20℃ for molecular analysis.

3.2.4 Determination of AMF colonization of roots by microscope

The ethanol preserved roots were washed under a steam of water and cut into 1 cm section, as described by Vierheilig et al. (1998). The excised roots were placed in 50 mL centrifuge tubes containing 10% (w/v) KOH and heated (90 ℃, 15 min) to remove the cytoplasmic contents and clarify the cortical tissue. Then the roots were washed with tap water after which blue ink was added to stain the fungal structures contained within the roots. The samples were again heated (90 °C, 30 s) and stored in lactoglycerol solution (1:1:1; lactic acid, glycerol and water).

Mycorrhiza root colonization rates were quantified by microscopy. For each root sample, three slides were made, containing ten ~1 cm root length segments per slide. In total 768 slides were analysed in microscope (30 segments in three slides per sample). Grades were assigned according to the amount of fungal structures, as described by Trouvelot (1986).

3.2.5 DNA extraction and PCR for mycorrhiza

Based on the results the of AMF colonization from microscope, 26 samples of brachiaria and 24 samples of crotalaria were selected, regardless of the microbiome derivate from the soil manipulation or AMF specie inoculated, to contemplate a gradient of colonization, from 0 to the highest colonization observed in the sample (Table 2). DNA was extracted from root samples (kept in -20°C), using a DNeasy ™ Plant Mini Kit (Qiagen, Catalog No. 69104), according to the manufacturer instructions. To verify the DNA extraction an 1.2% w/v agarose to 1× TAE buffer gel was run (Figure 1A).

From the DNA extract, a PCR amplification for mycorrhiza was procedure by using the FLR3 (5'-TTG AAA GGG AAA CGA TTG AAG T-3') and FLR4 (5'-TAC GTC AAC ATC CTT AAC GAA-3') primers (Gollotte et al. 2004) following the reaction: A 30 µL reaction mix contained PCR Buffer (Mg^{2+} free) (100nm Tris-Cl (pH8.8), 500nM KCl, 1% Triton-X-100) (2 µL) (Sinapse), 1 mM MgCl (2 µL) (Sinapse), 10mM dNTPs (2mM each) (1.56 µL) (Invitrogen), 0.01 mM primer (0.1 µL each), 5 U Taq polymerase (1 µL) (Sinapse), and ultrapure water, to complete the volume of the reaction (19.25 µL). An aliquot (1 µL) of DNA extract was added to 29 µL PCR mix. The PCR program was as follows: 93ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min (35 cycles), followed by 72ºC for 10 min. To verify the PCR products was run on 1.2% w/v agarose to 1× TAE buffer gel (Figure 1B).

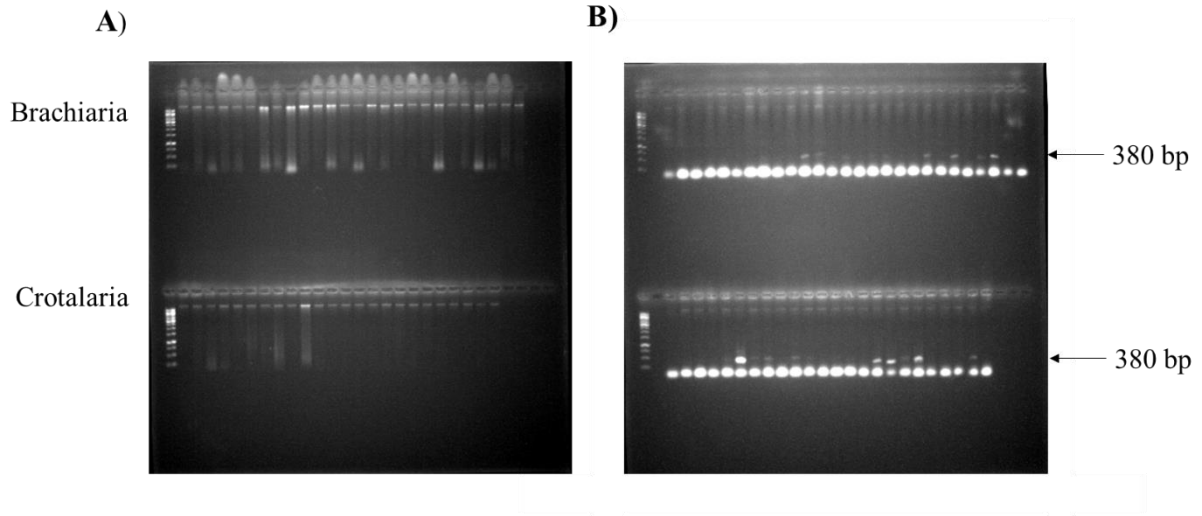

Figure 1. A) DNA extraction from root samples of brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*Crotalaria juncea* L.) (1.2% w/v agarose to 1× TAE buffer gel; 1kB ladder); **B)** PCR for mycorrhizal using the primers FLR3 and FLR4 (380 bp) from DNA samples of brachiaria (*U. brizantha*) and crotalaria (*C. juncea*) (1.2% w/v agarose to 1× TAE buffer gel; 1kB ladder).

Table 2. Mycorrhiza colonization of brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*C. juncea* L.) cultivated in a soil submitted to distinct manipulated microbiome. Plants were inoculated with: *Acalouspora colombiana*; *Rhizophagus clarus*; and *Dentiscutata heterogama*

AMF	Soil manipulation	Mycorrhiza colonization (%)	AMF	Soil manipulation	Mycorrhiza colonization (%)
----------Brachiaria----------			----------Crotalaria----------		
A. colombiana	SS+10^{-3}	0	*R. clarus*	S100	0
A. colombiana	S100	1	*R. clarus*	S100	0
A. colombiana	S100	1	*R. clarus*	S100	1
A. colombiana	S100	2	*R. clarus*	S50	5
A. colombiana	NS	3	*R. clarus*	S50	6
A. colombiana	S80	4	*D. heterogama*	SS	10
A. colombiana	S80	5	*D. heterogama*	S80	12
A. colombiana	S80	6	*R. clarus*	NS	15
A. colombiana	S50	6	*D. heterogama*	SS+10^{-3}	18
R. clarus	NS	12	*R. clarus*	S50	20
A. colombiana	S80	14	*R. clarus*	NS	20
A. colombiana	NS	17	*A. colombiana*	NS	24
D. heterogama	S80	18	*R. clarus*	S80	26
A. colombiana	S100	23	*A. colombiana*	SS+10^{-1}	28
D. heterogama	NS	24	*R. clarus*	S100	30
A. colombiana	S50	26	*A. colombiana*	SS+10^{-1}	32
No inoculated	NS	29	*A. colombiana*	SS+10^{-6}	36
R. clarus	S50	32	*D. heterogama*	SS+10^{-1}	38
R. clarus	NS	35	*D. heterogama*	SS+10^{-6}	40
A. colombiana	S50	39	*R. clarus*	S80	45
D. heterogama	NS	41	*A. colombiana*	S50	48
No inoculated	S50	43	*D. heterogama*	S50	52
A. colombiana	NS	47	*D. heterogama*	SS+10^{-6}	59
A. colombiana	S50	48	*A. colombiana*	S50	64
A. colombiana	NS	61			
D. heterogama	S50	65			

Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 50ºC for 1 h (S50), soil submitted to 80ºC for 1 h (S80); soil submitted to 100ºC for 1 h (S100); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) (SS+10^{-1}); (SS+10^{-3}); (SS+10^{-6}); and without dilution inoculation (SS).

3.2.6 Standard curve for qPCR

The PCR products of the three samples that amplified more, according to the agarose gel (Figure 1B) were purified using Wizard ™ SV Gel and PCR Clean-Up System Kit (Promega), following the manufactory instruction. To verify the PCR product purified, an 1.2% w/v agarose to 1× TAE buffer gel was run (Figure 2A).

A quantification of the product was made by using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific). The purified PCR product was used to prepare the curve by serial dilution:10^{-3}; 10^{-4}; 10^{-5}; 10^{-6}; 10^{-7}; 10^{-8}; 10^{-9}. Curve for qPCR was run following the reaction: A 30 µL reaction mix contained SYBR® Select Mix (10 µL) (Applied Biosystems, Life technologies), 0.02 mM primer (FLR3 and FLR4) (0.2 µL each), 10 mg L^{-1} BSA (0.3 µL) (Invitrogen), and ultrapure water, to complete the volume of the reaction (8.3 µL). An aliquot (1 µL) of each dilution of the curve was added to 29 µL PCR mix. The qPCR program was as follows: 94ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min (40 cycles), followed by 72ºC for 1 min. The melt curve, in the end of the reaction, variated from 60°C to 94°C. qPCR product of the curve was run on 1.2% w/v agarose to 1× TAE buffer gel (Figure 2B).

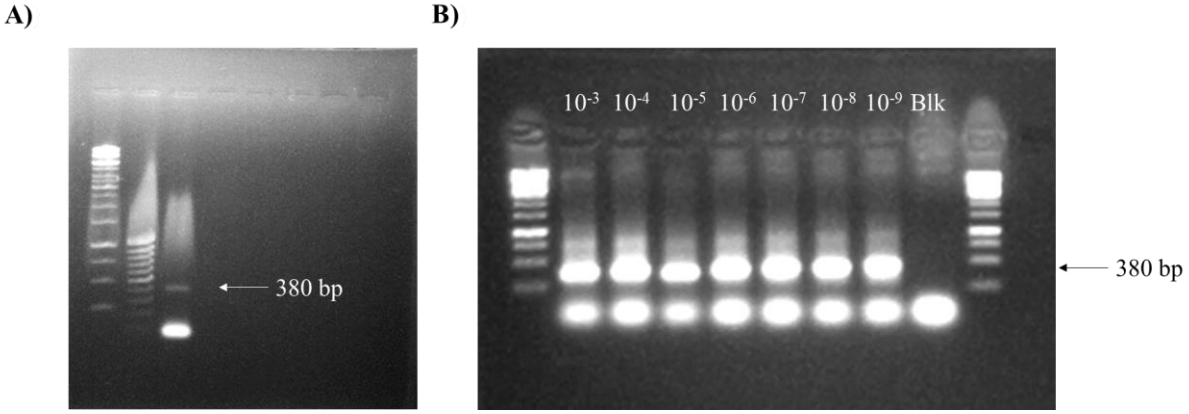

Figure 2. A) Purified PCR product for mycorrhizal using the primers FLR3 and FLR4 (380 bp) from DNA samples of brachiaria (*U. brizantha* cv. Marandu) and crotalaria (*C. juncea* L.) (1.2% w/v agarose to 1× TAE buffer gel; 1kB ladder and low mass ladder). **B)** qPCR curve product for a curve using the primers FLR3 and FLR4 (380 bp) from purified PCR product diluted (1.2% w/v agarose to 1× TAE buffer gel; 1kB ladder). Blk (Blank)

The copy number estimation of the gene per gram of root was calculated based on the quantification of purified PCR product (38.75 ng μL^{-1}) and the threshold cycles (CT) generated by the linear equation (y = -3.4732x + 32.0761; R^2=0.9999) using the dilutions of the gene 10^{-3} to 10^{-9}. The efficiency (Ef) of the qPCR was calculated as follow: $E = 10^{-1/slope} - 1$ (Park and Crowley 2005) (Figure 3).

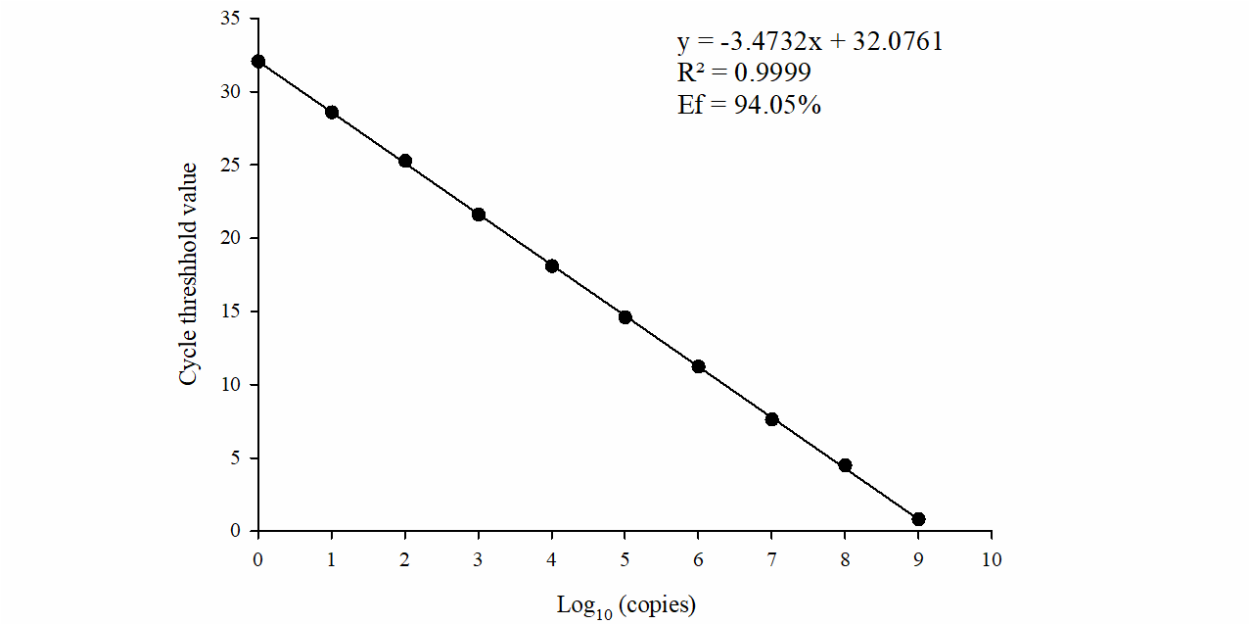

Figure 3. Standard curve for mycorrhiza from purified PCR product using FLR3 and FLR4 primers (380 bp).

3.2.7 qPCR from DNA samples of brachiaria (*U. brizantha* cv. Marandu) and crotalaria (*C. juncea* L.)

From the DNA extracted from the root samples of brachiaria and crotalaria, a qPCR amplification for mycorrhiza was procedure by using the same primers, FLR3 and FLR4, and the same reaction as described for the qPCR curve.

3.2.8 Statistical analysis

Correlation between the percentage of colonization obtained by microscope analysis and the log copy obtained by qPCR was made and the adjustment of the regression was obtained for all the data together, and separately per crop: brachiaria or crotalaria; and per AMF inoculated specie: *Acalouspora colombiana, Rhizophagus clarus or Dentiscutata heterogama.*

3.3 Results and discussion

Considering brachiaria and crotalaria together, the correlation coefficient (r) between the abundance of the gene by qPCR and the mycorrhiza colonization obtained by microscope was 0.73, and showed a R^2=0.526 (Figure 4), indicating that the qPCR technique is promising to complement microscopic analysis.

However, many factors may influence this correlation. An important factor is that the root samples were split in two subsamples at harvest, due to the different preparation required for each analysis. For microscope the roots are bleached and stained, and for qPCR the samples are submitted to DNA extraction. Redecker (2000) used the same dyed sample to extract the DNA, however, in a short scale, using 1-2 cm of root, according to the molecular scale, which may not be representative considering the number of root segments needed for qualification on microscope of a root sample.

Even coming from the same plant, the microscopic analysis and the molecular analysis were not run in the same material. Despite the root be equative separated, the heterogeneity of the sample may affect the correlation between the analysis. Gamper et al. (2008) related that root colonization is also spatially heterogeneous and although real-time PCR can reproducibly and accurately quantify AMF nucleic acids, these are poorly correlated with visual measures because of spatial heterogeneity.

Another point is related to the microscope analysis, which depend on human decision, to determine the values that represent the colonization, which should be standardized, but differences are likely to occur depending on the knowledge and experience of the evaluator. In this sense, Brundrett (2009) provide detailed protocols designed to analyse consistently AM in the microscope. Therefore, the same numeric results from the same samples, analysed by different evaluators are likely to be different, and so affect the correlation with the qPCR results.

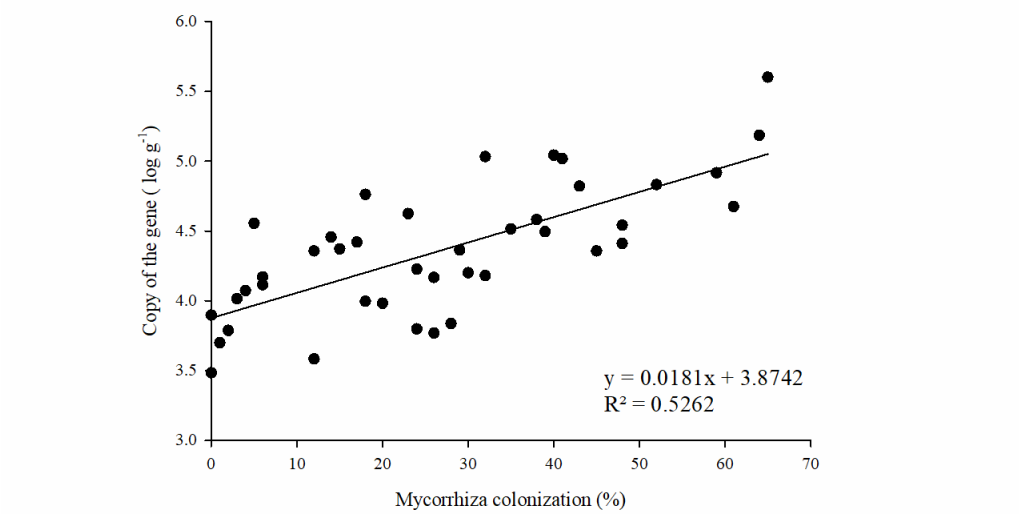

Figure 4. Correlation between the abundance of the gene for mycorrhiza, using FLR3 and FLR4 primers in a qPCR reaction, and mycorrhizal colonization (%), using microscope in roots of brachiaria (*U. brizantha* cv. Marandu) and crotalaria (*C. juncea* L.)

Analysing the correlation separately for each crop, a better correlation between microscope and qPCR analysis was observed for brachiaria compared to crotalaria. The regression adjustment between the abundance of the mycorrhiza gene and percentage of mycorrhiza for brachiaria showed a determination coefficient $R^2 = 0.6719$ (Figure 5A) When the correlation coefficient (r) was calculated for brachiaria data, r = 0.82, indicating a strong positive correlation. On the other hand, crotalaria showed lower explanation, about 34% (Figure 5B), and the correlation coefficient (r) was 0.58.

This result indicates that, even in the same microscope set of analysis and qPCR, differences among root samples may be an interference that may determine the correlation. Results of previous chapter of this thesis showed that the colonization rate evaluated in the microscope for brachiaria and crotalaria were different, even grown under the same soil conditions. Furthermore, according to the taxonomy, brachiaria, a Poaceae specie, has a threadlike root and absent of a taproot, whereas crotalaria, a Fabaceae specie, present a pivoting root system. The combination of those morphological factors may affect the distribution of colonization of the root system, and so reduce the accuracy of mycorrhiza analysis, both in microscope and qPCR.

Alkan et al. (2004) found a good correlation between the microscope and qPCR analysis, using a set of primers designed specifically for tomato, and so these authors highlighted the specificity of the qPCR for the crop in question.

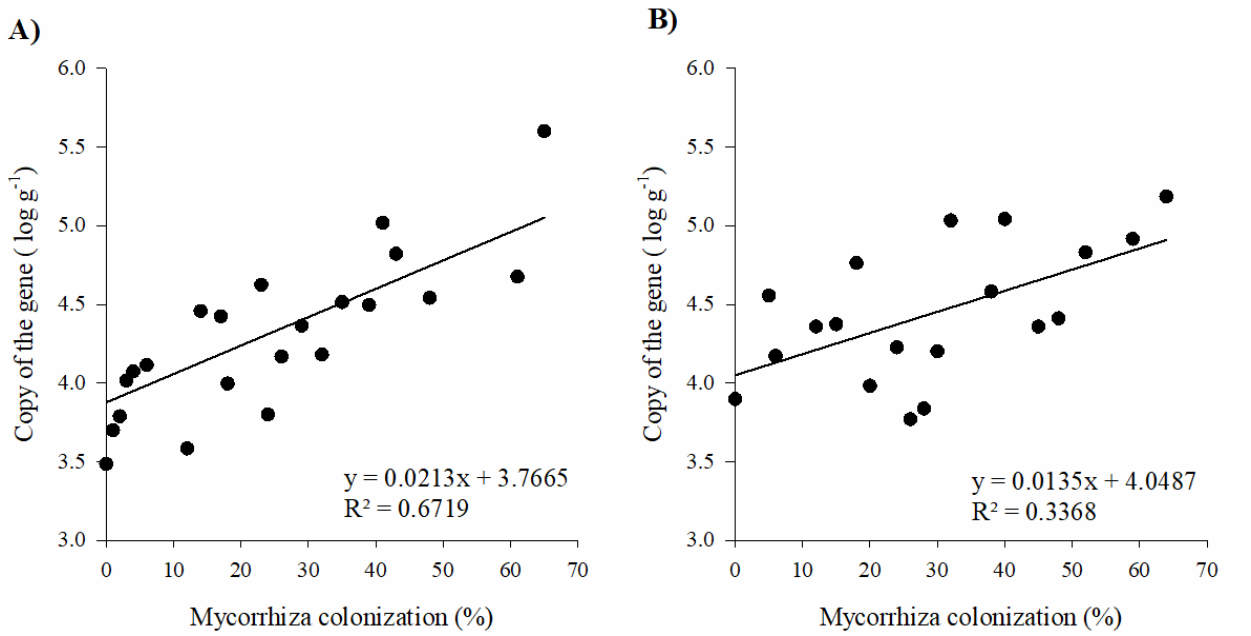

Figure 5. Correlation between the abundance of the gene for mycorrhiza, using FLR3 and FLR4 primers in a qPCR reaction, and mycorrhizal colonization (%) in roots of **A)** brachiaria (*U. brizantha* cv. Marandu) and **B)** crotalaria (*C. juncea* L.)

Separating the data according to the AMF species inoculated, *A. colombiana* and *D. heterogama* showed the same correlation, r=0.76, and very similar $R^2 = 0.5793$ and $R^2 = 0.576$ (Figure 6A, C). For *R. clarus* inoculation, the correlation between the abundance of the gene for mycorrhizal and the colonization was the lowest, r = 0.26, and $R^2 = 0.0668$ (Figure 6B).

Studies have shown that the set of primer may select and be more accurate for a specific group of mycorrhizas. Therefore, even with a high correlation for some mycorrhiza species between microscope and qPCR analysis, few species may not respond in the same level. Jansa et al. (2008) evaluated the mycorrhiza using both microscope and qPCR analysis (LR1 and FLR2 primers) in medic (*Medicago truncatula*) and leek (*Allium porrum*), inoculated with AMF, and observed a good correlation for some AMF species, however *G. mosseae* showed an opposite colonization results comparing the microscope and qPCR methodologies. These authors attributed this result to the vitality of the colonization, because microscope observation estimates the root colonization including both living and dead fungus, and are related to root length rather than root weight.

In a soil condition, even if spores of a specific AMF group are inoculated, the endogenous spores presented in the soil can colonize the roots, which makes difficult to predict the fungi that are colonizing the roots or not, to select the specific set of primer. Thonar et al.

(2012) studied the establishment of qPCR toolbox using the combination of specific primers for the rapid and specific quantification of 19 AMF isolates. These authors designed the markers that were validated using root samples from a field (maize, medicago, leek). As a result, the correlation between the methodologies for some AMF species was not observed, most likely, due to the presence of other AMF taxa, for which qPCR markers are not yet available (e.g. Acaulospora, Entrophospora, etc.).

3.4 Conclusion

The set of primers FLR3 and FLR4 was promising to correlate qPCR and microscope analysis, showing better correlation for brachiaria compared to crotalaria, and under inoculation of *A. colombiana* and *D. heterogama*.

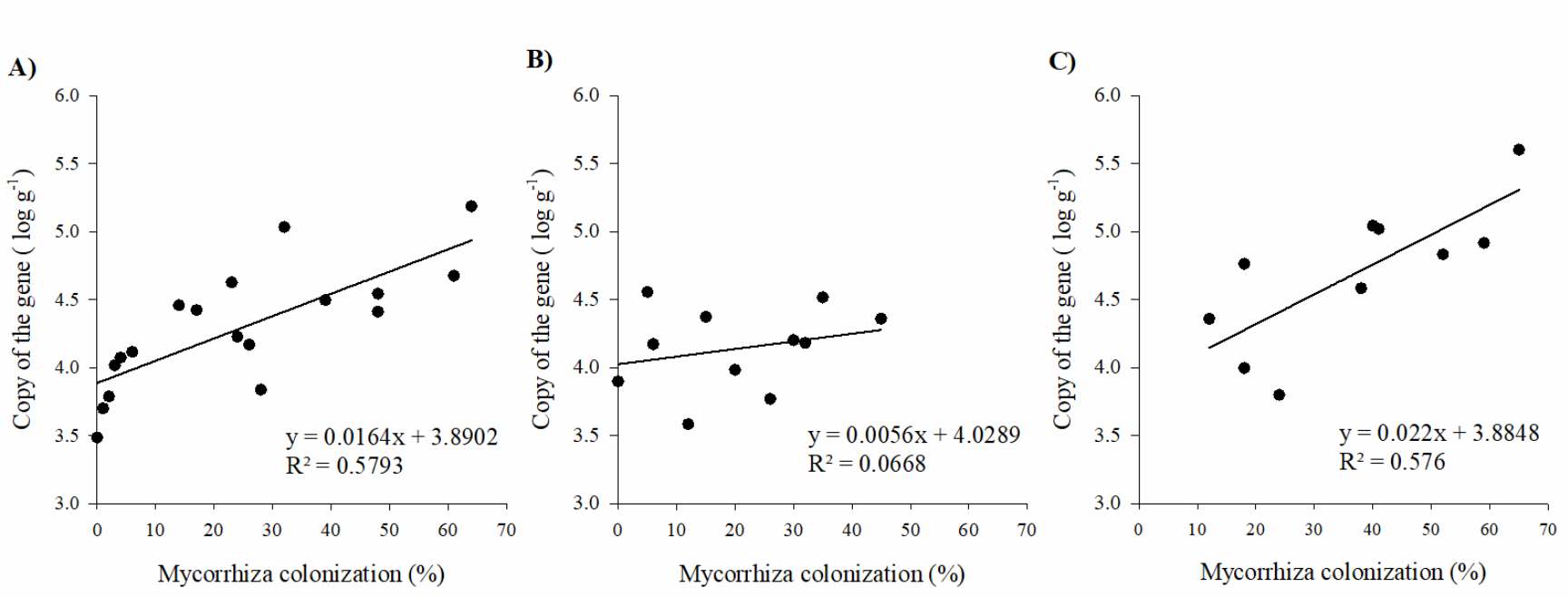

Figure 6. Correlation between the abundance of the gene for mycorrhiza, using FLR3 and FLR4 primers in a qPCR reaction, and mycorrhizal colonization (%) in roots of brachiaria (*U. brizantha* cv. Marandu) and crotalaria (*C. juncea* L.) inoculated with spores of arbuscular mycorrhiza fungi species: **A)** *Acalouspora colombiana;* **B)** *Rhizophagus clarus;* **C)** *Dentiscutata heterogama*

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4 PHOSPHORUS CYCLING IN SOILS WITH DISTINCT MANIPULATED MICROBIOME AND PHOSPHATE APPLICATION

Abstract
The hypothesis tested here is the occurrence of a compensation between the microbial diversity and the level of P suitable for plant uptake in the soil. The objective was to evaluate the P extraction by two plant species brachiaria *(Urochloa brizantha* cv. Marandu) and crotalaria (*Crotalaria juncea* L.) cultivated in soils hosting distinct microbial communities and P application. The experiment was conducted in glasshouse, where pots were used containing soil from four distinct manipulated microbiome compositions: *i)* natural soil; *ii)* soil submitted to a heat treatment (80 ºC; 1h); *iii)* sterilized soil (SS) by autoclaving (121 ºC; 2h; 1 atm) followed by inoculation of dilution SS+10^{-3}; and *iv)* SS, without dilution inoculation. In each situation, a gradient for P availability was established by using P fertilizer (triple superphosphate - TSP) at rates of 0, 20, 40 and 60 mg kg^{-1}. For 20 days, the soil moisture was maintained, and changes in chemical features were assessed by shifts in the micro and macronutrients and determination of the acid phosphatase activity (AP_{ASE}). After the microbial community stabilization, seedlings of brachiaria or crotalaria were transplanted to the soil. In a period of 30 days after the transplanting, inorganic labile P, microbial P (P_{MIC}), acid phosphatase activity (AP_{ASE}), and shoot P uptake (P_{UP}) were assessed. As results, brachiaria and crotalaria showed different trends. Brachiaria was more effected by the microbiome, and when cultivated under natural soil showed higher AP_{ASE} and P_{MIC}; however, higher shoot dry matter accumulation and P uptake were observed when brachiaria was cultivated under SS+10^{-3} and SS. On the other hand, crotalaria was not affected in the same level as brachiaria for all parameters, showing a neutral effect for the microbial manipulation. Brachiaria and crotalaria are generally used as cover crop and green manure and our results show a potential to use this species in degraded area without loss in plant biomass production. Furthermore, P application did not compensate the high microbial competition effect in a manipulated soil.

Key words: Diversity extinction; acid phosphatase activity; microbial P, shoot P uptake

4.1 Introduction

In highly weathered tropical soils, phosphorus (P), a macronutrient required to the development of the plants, is strongly adsorbed on the surface of sesquioxides, such as aluminium oxide, which reduces its availability to the plants (Parfitt 1979).

To provide P to the plants in an agroecosystem, fertilizer application, such as triple superphosphate, is the most used strategy (Sample et al. 1980). However, even when P is applied, the adsorption process reduces its availability to the plants very quickly (Raghothama and Karthikeyan 2005), decreasing the mobility of P in the soil and lacking the contact between phosphate and roots for absorption (Rausch and Bucher 2002). Another fact to consider is that

apatite rocks, main mineral source for P fertilizer, are a finite resource, and so must be applied in a sustainable way, to guarantee the food security in the present and future.

Furthermore, many microorganisms are responsible to determine the P availability to the plants, by acting in different process such as mineralization and solubilization of P (Kumar et al. 2018). Microbial community can either improve the plant P uptake or compete by this nutrient in the soil. In this sense, microbial community may modify the sinks and availability of the P in the soil, and thus the P uptake by plants.

Therefore, phosphorus use efficiency is dependent on soil properties, microorganisms in the soil, and the plant characteristics, such as morphological roots that may become a strategy of soil exploration for P uptake (Machado and Furlani 2004). In addition, plants have strategies to select organisms in the rhizosphere, however, the selected organisms may have either positive or negative effects for the plant (Tkacz et al. 2015).

Although some mechanisms evolving the regulation of phosphorus uptake and utilization have been studied, more efforts are required to further articulate the mechanisms controlling P uptake and utilization within plant (Hasan et al. 2016). Thus, strategies that evolve plant, microorganism and fertilizer application should be coordinated to guarantee the sustainability of the agroecosystem (Adesemoye and Kloepper 2009).

The hypothesis tested here is the occurrence of a combinatory effect between microbial diversity and level of P applied in the soil on plant P uptake. The objectives of this work were: i) to verify if the loss of microorganisms caused by different extinction methods would affect the plant P uptake; ii) to investigate the compensation effect of the levels of P applied and levels of soil microbial diversity to harness the P uptake by plants.

4.2 Material and methods

4.2.1 Experimental setup

The experiment was conducted in a glasshouse (27°C ± 3), in a factorial design (4 x 4), with four replicates. Each experimental unit comprised a plastic pot (5 cm internal diameter × 9 cm tall), containing 270g of soil (dry weight). Soil was classified as a sandy clay loam textured Ferralsol (WRB-FAO, 2015), collected from a depth of 0-20 cm within a livestock-grazed

pasture, in Piracicaba, Sao Paulo, Brazil (22°43’03.0’’S, 47°37’00.4’’W). The main characteristics of this soil is presented in table 1, as natural soil (NS), 0 P application.

4.2.2 Manipulation of soil microbiome and P application

The soil was treated to generate four different microbiomes: i) natural soil (NS), used as untreated and positive control; ii) soil submitted to heat shock by using 80ºC for 1 h (S80) (Voort et al. 2016); iii) sterilized soil (SS) by autoclaving twice (121 ºC, 103 kPa, 1 h) following the serial dilution inoculation SS+10^{-3} (v/w of natural soil dilution added to the sterile soil); and iv) SS, without dilution inoculation, as a negative control (van Elsas et al. 2012).

In the same day, after the microbiome was manipulated, P fertilizer as triple superphosphate (TSP; 45% soluble P_2O_5) was applied at rates: 0; 20; 40 and 60 mg kg^{-1}, based in soluble P, corresponding to: 0; 40; 80 and 120 kg ha^{-1}. The doses were based on 0; 50%; 100% and 150% of the recommendation to the farmers for brachiaria and crotalaria, according to the manual of recommendations to Sao Paulo state (Raij et al. 1997).

4.2.3 Monitoring the microbial and P treatments

After the microbial and P treatments setup, the soil moisture was kept in 70% of maximum hold capacity (60 mL pot^{-1}), using deionized and sterilized water to promote cell multiplication of the differentiated communities, matching the microbial abundance in all treatments. After 20 d of the treatments setup, a soil sampling was made for chemical analysis (macro and micronutrients) (soil sample was air-dried) and acid phosphatase activity (AP_{ASE}) (soil sample was kept in 4℃ until analysis).

From the air-dried soil sample were determined (Raij et al. 2001): macronutrients (P; S; K; Ca; Mg); micronutrients (B; Cu; Fe; Mn; Zn) acidity attributes (pH_{CaCl2}, Al; H+Al) and the organic matter, for soil characterization (Table 1). Additionally, inorganic labile P was determine using anion exchange resin (P_{AER}) and $NaHCO_3$ (P_{NaHCO3}) (Murphy and Riley 1962).

Acid phosphatase activity (AP_{ASE}) analysis was performed according to Tabatabai and Bremner (1969). For $AP_{ASE,}$ statistical analysis was made by comparison of the interaction between the microbiome and the rates of P using analysis of variance (ANOVA) considering significance ($p \leq 0.05$) by F values. Comparison of the interaction, among soil manipulation

treatment was performed using the LSD test ($p \leq 0.05$) and differences among rates of P was made by regression adjustment.

Table 1. Chemical analysis of a soil samples, collected in the 0-20 cm layer in Piracicaba, Sao Paulo, Brazil, 20 days after soil manipulation (SM) and P rates application (PRA), before cultivation.

Treatments		pH	OM	P_{SR}	S	K	Ca	Mg	Al	H+Al	B	Cu	Fe	Mn	Zn
SM	PRA														
	mg kg^{-1}	$_{CaCl2}$	g dm^{-3}	----mg dm^{-3} ----		----------mmol$_c$ dm^{-3}----------								-----g kg^{-1} -----	
NS	0	5.0	38	10	11	<0.9	16	7	1.00	25	<0.15	0.8	77	5.8	3.2
NS	20	5.1	39	12	7	<0.9	15	8	1.00	22	0.19	0.9	79	5.5	3.6
NS	40	5.0	41	14	<6	<0.9	15	8	<0.02	16	0.18	0.9	78	6.4	3.5
NS	60	5.1	39	15	<6	<0.9	14	7	<0.02	25	0.17	0.9	85	5.9	3.7
S80	0	5.0	39	11	<6	<0.9	14	7	<0.02	25	0.15	0.8	79	7.0	3.7
S80	20	5.0	40	14	9	<0.9	15	7	1.00	28	<0.15	0.8	80	7.8	3.6
S80	40	5.0	42	17	7	<0.9	15	7	1.00	25	<0.15	0.8	74	7.6	3.5
S80	60	5.1	40	19	9	<0.9	15	7	1.00	25	<0.15	0.8	77	5.8	3.6
SS	0	5.1	34	8	27	<0.9	17	8	<0.02	20	0.30	0.8	100	31.8	2.8
SS	20	5.1	39	11	17	<0.9	20	9	1.00	25	0.24	0.8	105	33.8	3.0
SS	40	5.3	33	10	47	<0.9	20	8	1.00	22	0.26	0.9	170	26.9	2.9
SS	60	5.2	34	14	30	<0.9	19	9	<0.02	22	0.32	0.8	122	31.5	2.9

Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS). Soil with dilution inoculation (SS+10^{-3}) was considered similar to SS regarding chemical analysis. Chemical analysis abbreviations are defined as follows: OM: organic matter; P_{SR}: P extracted by spherical resin

4.2.4 Pre-germination of plants, transplanting and cultivation

Brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*Crotalaria juncea* L.) seeds were disinfected with 2% (v/v) NaClO solution (15 min), and rinsing in sterilized deionized water. Germination was carried out 15 d after the soil was submitted to manipulation of soil microbiome and P application, and sown in a sterile and phosphorus-free sand. The seedlings were watered with sterilized and deionized water until the transplant to the pots.

The implantation of the experiment occurred 20 d after the manipulation of soil microbiome and P application treatments. The pre-seedlings (5 d old) were immersed in sterilized deionized water (30 min) to facilitate their removal from the sand. The pots with the newly transplanted seedlings remained in the room for 48 h with indirect light. Afterwards, they were taken to the glasshouse, where they remained for 28 d, until the experimental harvest. During this period, pots were irrigated with sterilized and deionized water.

4.2.5 Harvest of the experiment, soil and plant sampling and analysis

At the harvest time, the plants were removed from the pots for the shoot and soil sampling. The shoot was dried in the oven at 55°C until stabilize the weight to obtain the dry matter. After, the dried shoot was individually triturated in liquid nitrogen with a ceramic crucible support and pistil, later sieve through 150 µm mesh and 30 mg were used for the P analysis. P content was determined in the Nuclear Instrumentation Laboratory, at CENA/USP, using Energy-dispersive X-ray spectroscopy (EDX) technique.

Part of the soil was kept in a cold chamber (4ºC) for analysis of acid phosphatase activity enzyme (AP_{ASE}) (Tabatabai and Bremner 1969) and microbial P biomass (P_{MIC}) (Brookes et al. 1984). Another part of the soil was air dried to analyse of inorganic labile P fractions extracted with anion exchange resin (P_{AER}) and $NaHCO_3$ (P_{NaHCO3}) (Murphy and Riley 1986).

For AP_{ASE}, P_{MIC}, P_{AER} comparison of the interaction between the microbiome and the rates of P was made by analysis of variance (ANOVA) considering significance ($p \leq 0.05$) by F values. Comparison of means among soil manipulation treatment was performed using the LSD test ($p \leq 0.05$) and differences among rates of P was made by regression adjustment. Statistical analysis was performed separately for brachiaria and crotalaria. Principal component analysis was performed with brachiaria and crotalaria together.

4.3 Results

4.3.1 Effect of the microbiome manipulation and P application in inorganic labile P and acid phosphatase activity

After soil microbiome manipulation, a reduction in P_{AER} in the autoclaved soil was observed in comparison to the natural soil, however P_{NaHCO3} was higher in the SS, resulting in a compensation in the inorganic labile P, and no changes in the levels of labile P was observed between NS and SS. The changes observed were regarding to the P application, in a gradient according to the doses of P (Figure 1).

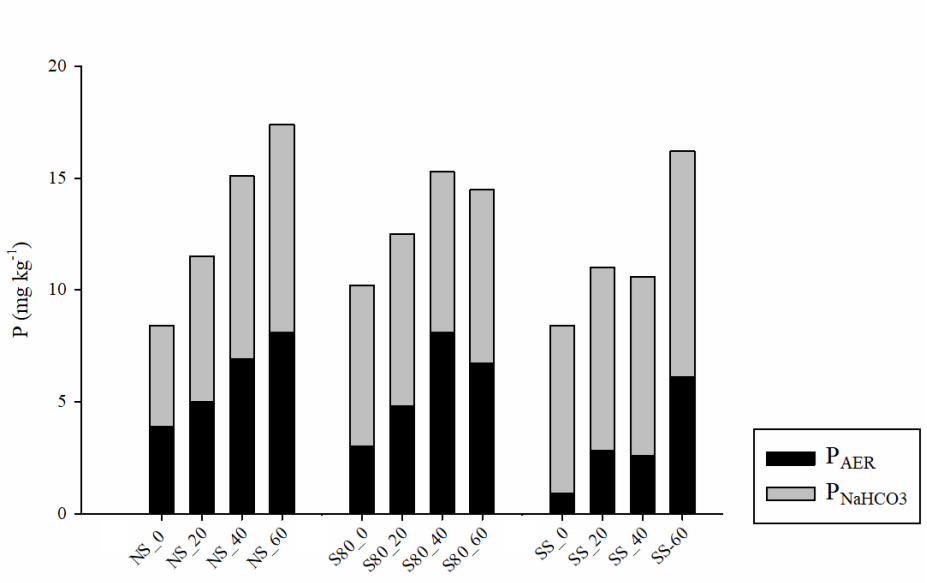

Figure 1. Labile P (P_{AER} + P_{NaHCO3}) 20 days after soil microbiome manipulation. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS). P fertilizer was applied as triple superphosphate at rates: 0; 20; 40 and 60 mg kg^{-1}. Chemical analysis abbreviations are defined as follows: P_{AER}: P extracted by anion exchange resin; P_{NaHCO3}: inorganic P extracted by $NaHCO_3$.

In general, the highest acid phosphatase activity (AP_{ASE}) was observed in the natural soil (NS) condition, compared to the S80, SS+10^{-3}, and SS, but P doses did not interfere in the activity in NS (Figure 2). For S80, when 60 mg kg^{-1} of P was applied, an increment of the AP_{ASE} was observed, showing a compensatory effect compared to NS. For treatments SS+10^{-3} and SS, the lowest activities of AP_{ASE} were observed when P was amended, and no compensation between the soil manipulation and P application for AP_{ASE} was observed (Figure 2).

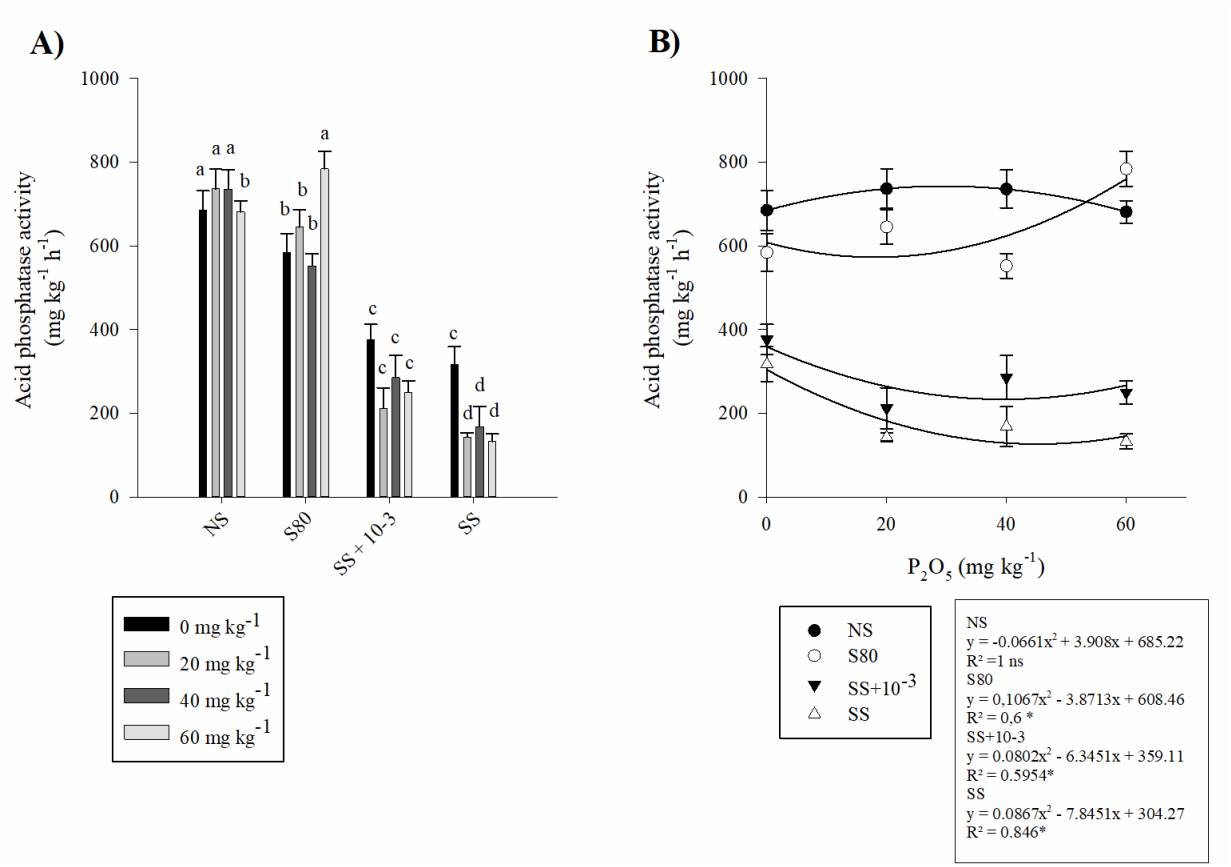

Figure 2. Acid phosphatase activity 20 days after soil microbiome manipulation. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-3}$); and without dilution inoculation (SS). P fertilizer was applied as triple superphosphate at rates: 0; 20; 40 and 60 mg kg^{-1}. **(A)** Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test. **(B)** Regression adjustments are related to P rates. * Significant ($p \leq 0.05$); ns: no significant ($p > 0.05$).

4.3.2 Effect of brachiaria and crotalaria in soil microbial features after microbiome manipulation and P application

For AP_{ASE}, after cultivation with both brachiaria and crotalaria, the highest activity was observed when the plants were cultivated under NS and S80, and the lowest activities were observed under SS+10^{-3} and SS (Figure 3A and B). The compensatory effect of P application was observed for brachiaria under 20 mg kg^{-1} application, where S80 equivalent AP_{ASE} compared to NS (Figure 3A). For crotalaria this compensatory effect between NS and S80 was observed under 60 mg kg^{-1} (Figure 3B).

For microbial P (P_{MIC}), brachiaria showed higher P_{MIC}, in magnitude, compared to crotalaria for all the treatments and for both brachiaria and crotalaria, the NS showed the highest P_{MIC} (Figure 4). Considering the P rates applied, for brachiaria, only NS and S80 showed effect for P application, where the P application reduced P_{MIC} in NS, and for S80 an opposite trend was observed, where the increment of P application increased the P_{MIC}, with a compensatory effect between NS and S80 under 60 mg kg^{-1} dose (Figure 4A). Crotalaria showed effect of P rates under NS and SS, and in both situations P application decreased P_{MIC} and therefore, no compensatory effect was observed (Figure 4B).

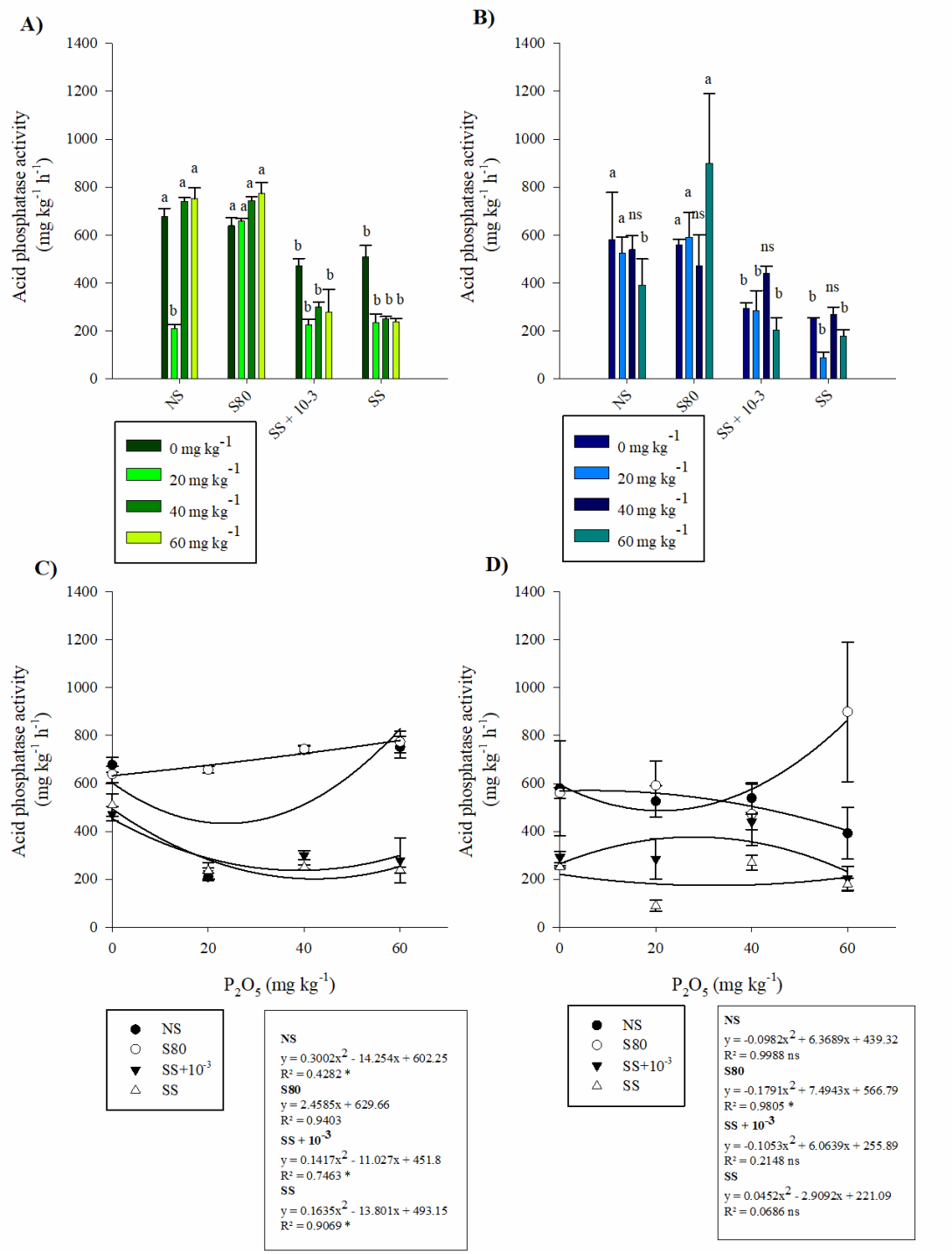

Figure 3. Acid phosphatase activity of soils cultivated with brachiaria **(A)** and crotalaria **(B)** subjected to distinct microbiome manipulations and P application. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-3}$); and without dilution inoculation (SS). P fertilizer was applied as triple superphosphate at rates: 0; 20; 40 and 60 mg kg^{-1}. **(A)** and **(B)** Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test. **(C)** and **(D)** Regression adjustments are related to P rates. * Significant ($p \leq 0.05$); ns: no significant ($p > 0.05$).

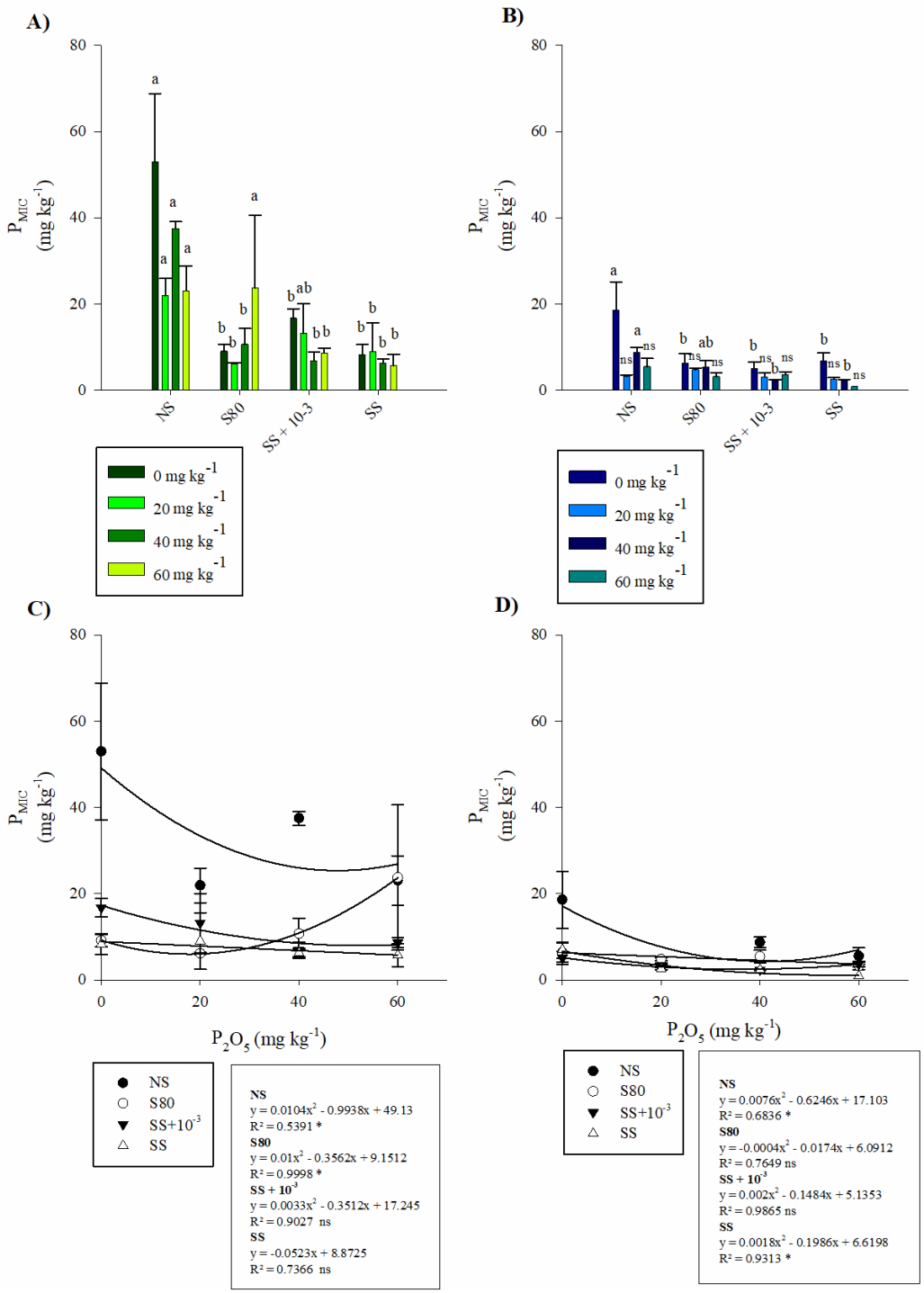

Figure 4. Microbial P (P_{MIC}) of soils cultivated with brachiaria **(A)** and crotalaria **(B)** subjected to distinct microbiome manipulations and P application. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-3}$); and without dilution inoculation (SS). P fertilizer was applied as triple superphosphate at rates: 0; 20; 40 and 60 mg kg^{-1}. **(A)** and **(B)** Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test. **(C)** and **(D)** Regression adjustments are related to P rates. * Significant ($p \leq 0.05$); ns: no significant ($p > 0.05$).

4.3.3 Effect of brachiaria and crotalaria in soil P labile fraction after microbiome manipulation and P application

In general, for crotalaria cultivation, more labile P was observed in the soil after cropping, compared to brachiaria (Figure 5). For brachiaria, the highest levels of P remained in the soil solution after cropping were observed under NS and S80, and the treatments SS+10^{-3} and SS showed the lowest P_{AER} remained in the soil solution after cropping. Regarding to the P levels application for brachiaria, P_{AER} was affected for all the microbiome manipulations treatment, where the labile P in the soil solution increased by the rates of P applied (Figure 5A).

For crotalaria, under no P application, a gradient in P_{AER} was observed, where NS showed the highest level, decreased with S80 and SS+10^{-3} and the lowest value was observed under SS. When 20 mg kg^{-1} was applied, no differences between the microbiome compositions were observed. And when 40 and 60 mg kg^{-1} were applied, the highest P_{AER} was observed under S80, followed by NS and SS+10^{-3} and the lowest value were observed in SS. Regarding the P rates, for all the microbiome manipulations, a significant effect was observed, where the lowest P_{AER} were observed under 40 mg kg^{-1}.

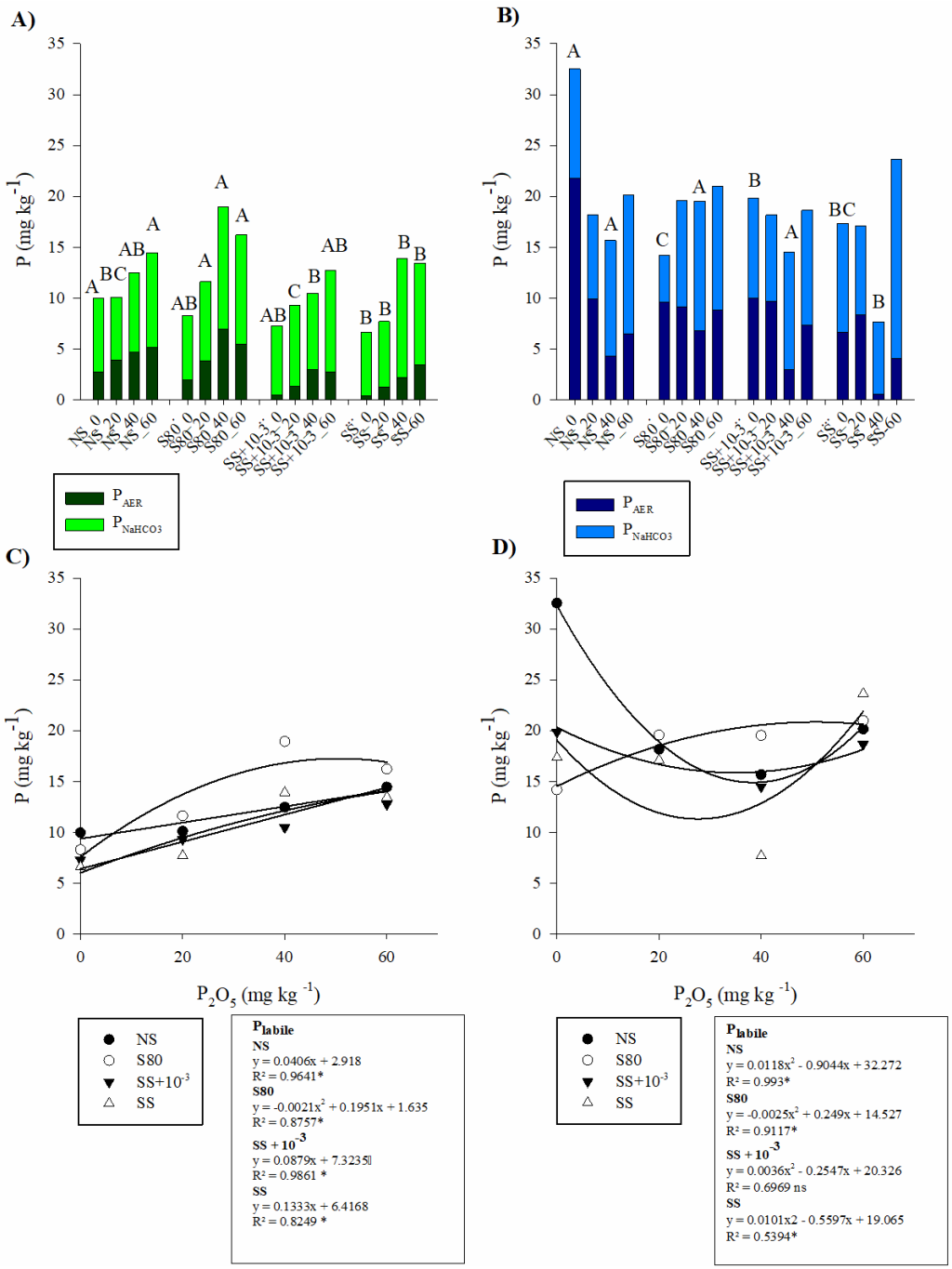

Figure 5. Labile P (P_{AER} + P_{NaHCO3}) of soils cultivated with brachiaria **(A)** and crotalaria **(B)** subjected to distinct microbiome manipulations and P application. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-3}$); and without dilution inoculation (SS). P fertilizer was applied as triple superphosphate at rates: 0; 20; 40 and 60 mg kg^{-1}. **(A)** and **(B)** Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test. **(C)** and **(D)** Regression adjustments are related to P rates. * Significant ($p \leq 0.05$); ns: no significant ($p > 0.05$). Chemical analysis abbreviations are defined as follows: P_{AER}: P extracted by anion exchange resin; Pi $_{NaHCO3}$: inorganic P extracted by $NaHCO_3$.

4.3.4 Dry matter and shoot P content of brachiaria and crotalaria cultivated under distinct microbiome manipulation and P application

For brachiaria, the shoot dry matter production and P uptake were higher when cultivated under SS+10^{-3} and SS, compared to the NS and S80 (Figure 6A and 7A). Regarding to the doses of P applied, a gradient was observed for all treatments, NS, S80, SS+10^{-3} and SS, where the increment in P applied increased the shoot dry matter and shoot P uptake, and therefore no compensatory effect by P application on microbiome manipulation.

For crotalaria, under no P application, no difference was observed in shoot dry matter production and P uptake between NS, S80, SS+10^{-3} and SS (Figure 6B and Figure 7B) and in general, the highest shoot dry matter production and P uptake were observed in SS, under 20 and 60 mg kg^{-1}.

Taking together the results of brachiaria and crotalaria, a clear distinction was observed between the crops, without effect of the P application, but the microbiome manipulation. Brachiaria under NS and S80 was related to high microbial feature, AP_{ASE} and P_{MIC}. High plant features, evolving shoot dry matter accumulation and P uptake were related to brachiaria cultivated under SS+10^{-3} and SS. On the other hand, Crotalaria was more related to high P_{AER} remain in the soil (Figure 8).

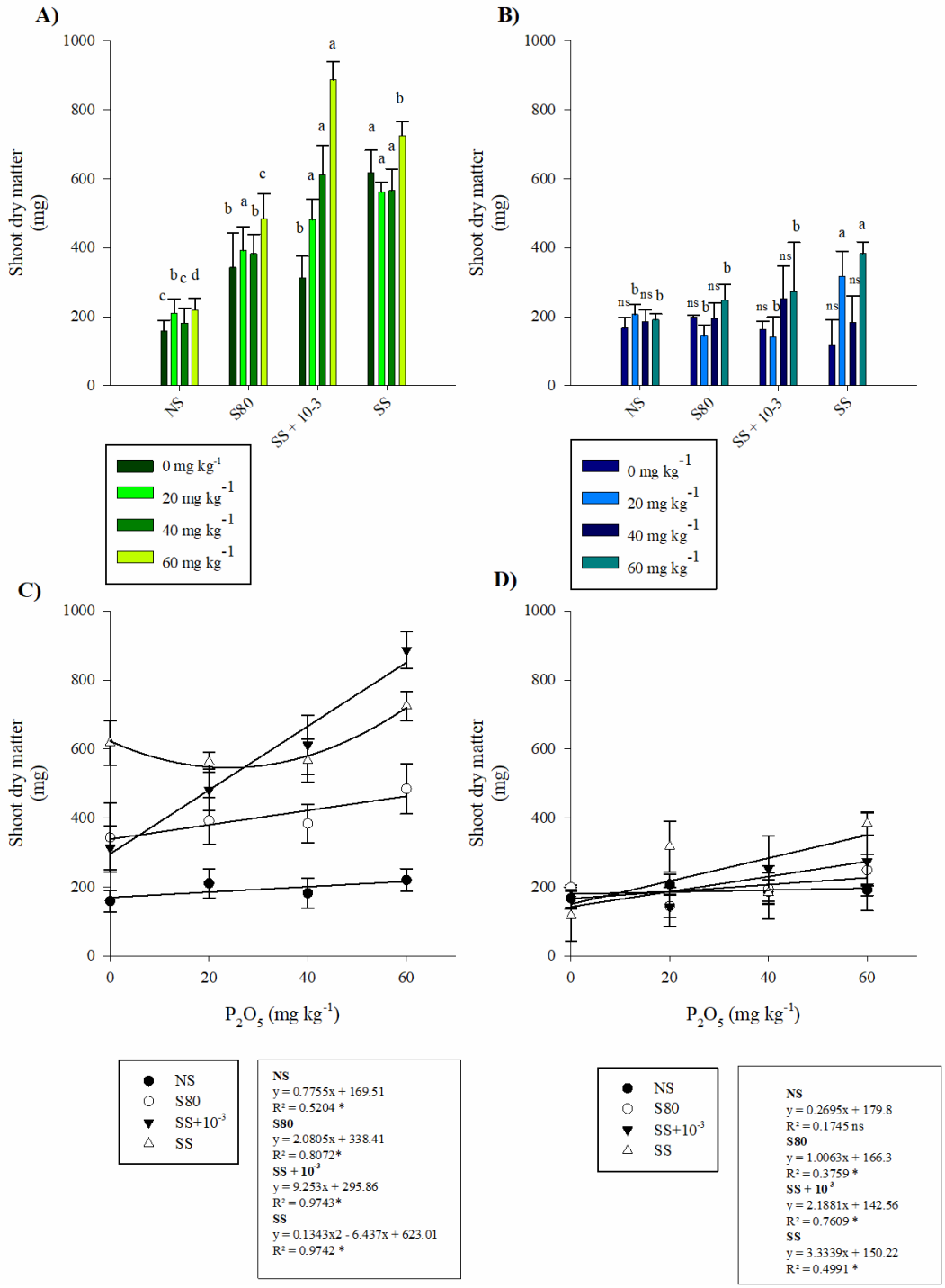

Figure 6. Shoot dry matter of brachiaria **(A)** and crotalaria **(B)** cultivated in soil subjected to distinct microbiome manipulations and P application. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-3}$); and without dilution inoculation (SS). P fertilizer was applied as triple superphosphate at rates: 0; 20; 40 and 60 mg kg^{-1}. **(A)** and **(B)** Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test. **(C)** and **(D)** Regression adjustments are related to P rates. * Significant ($p \leq 0.05$); ns: no significant ($p > 0.05$).

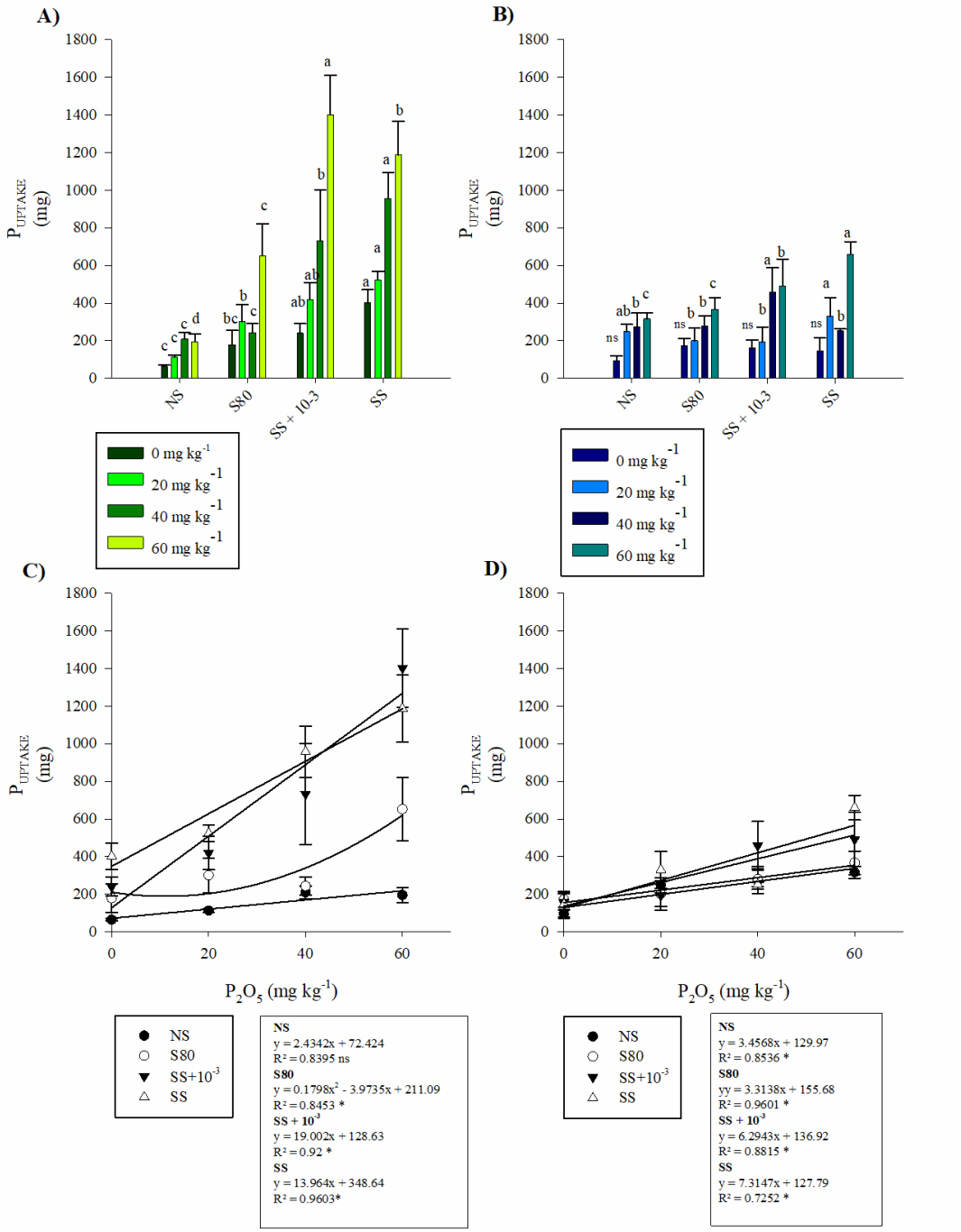

Figure 7. Shoot P uptake of brachiaria **(A)** and crotalaria **(B)** cultivated in soil subjected to distinct microbiome manipulations and P application. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) ($SS+10^{-3}$); and without dilution inoculation (SS). P fertilizer was applied as triple superphosphate at rates: 0; 20; 40 and 60 mg kg^{-1}. **(A)** and **(B)** Different letters show significant difference between soil manipulation treatments ($p \leq 0.05$) by LSD test. **(C)** and **(D)** Regression adjustments are related to P rates. * Significant ($p \leq 0.05$); ns: no significant ($p > 0.05$).

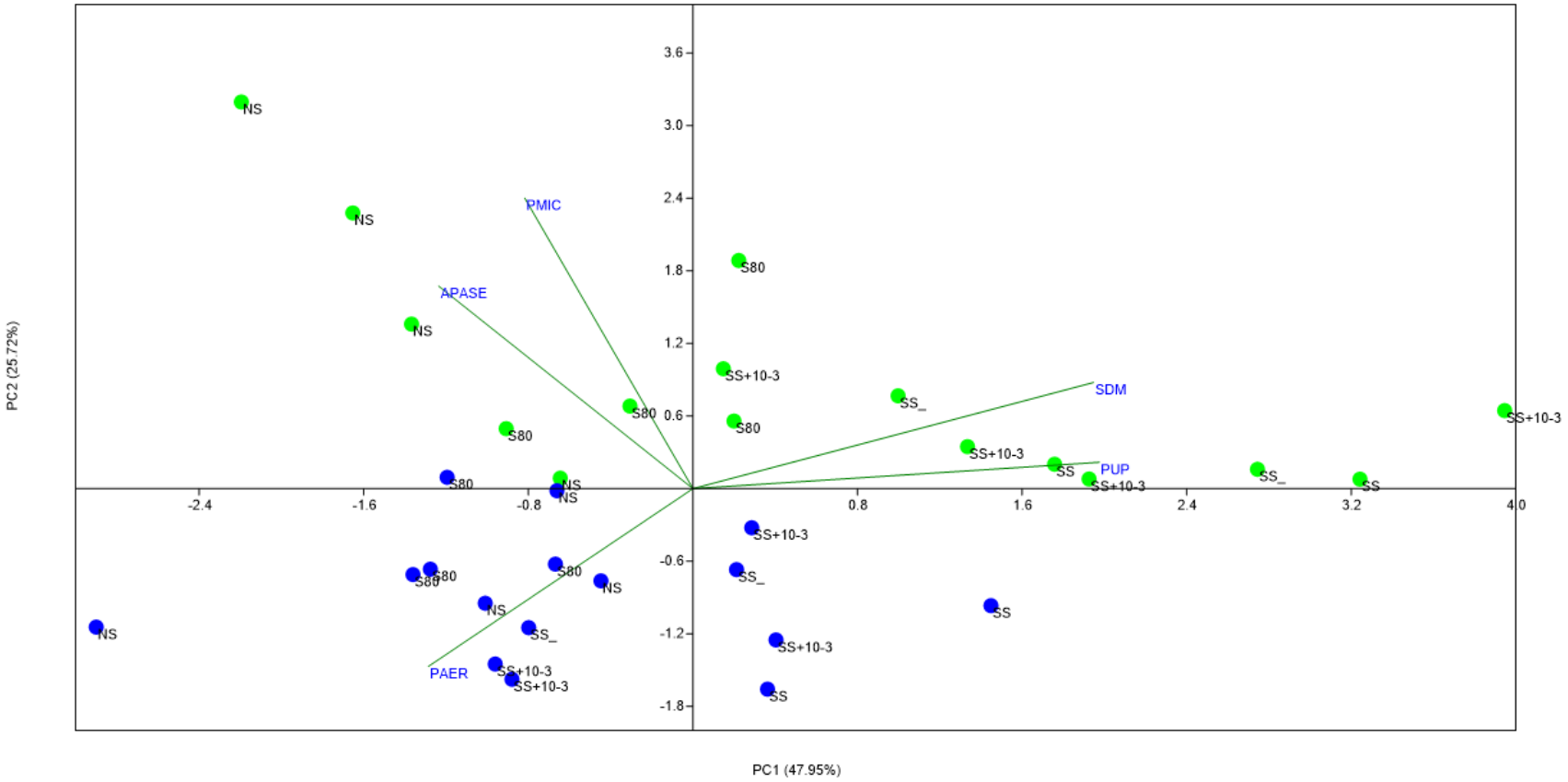

Figure 3. Principal components of brachiaria (●) and crotalaria (●) cultivated in soil subjected to distinct microbiome manipulations and P application. Treatment abbreviations are defined as follows: natural soil (NS); soil submitted to 80ºC for 1 h (S80); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS) followed by inoculation of dilution (10% v/w of natural soil) (SS+10^{-3}); and without dilution inoculation (SS). P fertilizer was applied as triple superphosphate at rates: 0; 20; 40 and 60 mg kg^{-1}.
PMIC: microbial P; PAER: inorganic labile P extracted using anion exchange resin; APASE: acid phosphatase activity; SDM: shoot dry matter; PUP: uptaken P from the shoot.

4.4 Discussion

4.4.1 Effect of the microbiome manipulation and P application in inorganic labile P and acid phosphatase activity

Changes promoted by the extinction methods were checked by inorganic labile P and AP_{ASE} activity. In general the amount of inorganic labile P was not changed by the methods used here to promote changes in the microbial community (heat shock or autoclave followed by re-inoculation), however a reduction in P extracted by resin was observed under SS compared to NS. Resin extracts P from the soil solution in a comparable system of the plant, simulating the roots (Amer et al. 1955). The reduction in resin P level come along with an increase in P_{NaHCO3} under SS, that represents the easily available P for the plant. Therefore, although inorganic labile P did not reduce by the changes in the microbial community, our results show that the labile phosphorus dynamics is affected by the microbial community manipulation, which may affect the fate of P in the soil system (Deubel and Merbach 2005).

A reduction in AP_{ASE} according to the level of microbial manipulation indicates a clear connection between this proxy and the differential composition of the soil microbiome. The same results were demonstrated in the previous chapter of this thesis. Serrasolsas and Khanna (1995) studied the phosphatase activity after autoclaving soils, and the results showed that right after the autoclaving no activity was detected, what was associated to the denaturalization of the enzymes, but a partial recovery in the activity was observed after seven months of incubation.

4.4.2 Response of brachiaria and crotalaria cultivated in soil after microbiome manipulation and P application

Our results showed that when brachiaria was cultivated in the natural soil (NS), without any extinction method, this crop specie was able to keep acid phosphatase activity (AP_{ASE}) in the soil in high levels, which was also observed in the previous chapter of this thesis.

Furthermore, under brachiaria cropping the microorganisms were able to incorporate P in their biomass as microbial P (P_{MIC}) in NS, which indicates high microbial activity in this system. In a high diversity condition, some microorganisms may take advantage in the P usage, resulting in high immobilization of available P, reducing its availability to the plants (Marschner 2008).

Additionally, higher levels of labile P remained in the soil solution were observed after brachiaria cultivation, under NS, indicating that this crop did not absorbed the available P from the soil.

Consequently, brachiaria cultivated under NS showed lower dry matter and P uptake than the manipulated soils, indicating that brachiaria was not able to compete against microorganisms, which resulted in low shoot dry mass and low P uptake. McLaughlin and Alston (1986) observed a decrease in dry weight and P uptake of wheat in the presence of ^{33}P-labelled residues of medic (*Medicago trunculata* cv. Paraggio) and concluded that this reduction was due to competition between the plants and soil biomass for available P. Also, Turner et al. (2013) observed that in mature ecosystems, soil microbes accounted for most of the total biomass phosphorus in comparison to plant phosphorus, and pointed out to the central role of the microbial biomass in determining phosphorus availability due to the strong competition between plants and saprotrophic microbes for soil phosphorus.

The opposite trend was observed when brachiaria was cultivated under soil submitted to extinction methods. The sterile soil condition showed a low AP_{ASE} and low P_{MIC}, and less P_{AER} remained in the soil after cropping with brachiaria. Taking all together, these factors resulted in high shoot dry matter accumulation and high P uptake for brachiaria when cultivated under SS. Li et al. (2019) observed higher growth of peach, corn and tomato in a sterilized soil compared to the natural soil and after cropping observed the presence of plant growth promoting rhizobacteria in the rhizosphere, additionally with reduction of pathogens by the sterilization of the soil, which was also observed by Sosnowski et al. (2009).

Another factor to take into account for this response is the crop itself. Brachiaria has a fire resistance in the field, as described by Nunes et al. (1984), and due to this capacity, even after an event of fire, that may harm and change the neighbour community by killing the sensitive organisms, brachiaria can resist and survive. Gorgone-Barbosa et al. (2015) described *U. brizantha* as a resistant specie to fire event, revelling that this crop survived even after fire event in Brazilian Cerrado, tolerating temperatures higher than 600 °C more often than the native grass. This characteristic may favour brachiaria in a stressed condition, such as autoclaved soil, where the microbial community may be devastated, but brachiaria can develop well after the changes promoted by the stress.

Crotalaria showed less dependency on the treatments that changed the microbial community, for both aspects, shoot dry matter accumulation an P uptake response, and the magnitude of the response was lower compared with brachiaria, indicating that the microorganisms and P application were not strongly determinant for crotalaria development. Similar results were obtained in the previous chapter of this thesis, that showed low dependency of crotalaria to the microbial community for mycorrhizal colonization.

4.4.3 Effect of microbiome manipulation on plant

Our results showed that even after a perturbance in the soil, brachiaria was favoured by the reduction of diversity and crotalaria was not affected in biomass production and P uptake indicates that the response to the reduction of diversity depend on the crop. Such evidences might rely on the fate that plants affect the rhizosphere, thus the microbiome vicinity and P availability, shaping the conditions according to the needs (Berg and Smalla 2009; Kowalchuk et al. 2002; Marschner et al. 2001).

The level of degradation that occur in the area will determine how it will affect the microbial community and so, the plant. Depending on the microorganisms extinguished of the system after manipulation or degradation, the competition between the plants and the survivor microorganisms may be reduced and more P will be available for plant uptake. Li et al. (2019) demonstrated that rhizosphere community of a non-native plant cultivated in soil sterilization can be rapidly recovered with the influence of the plant and the new microbial community that develops is a healthier rhizosphere microbiome.

Besides that, beneficial microorganisms, that may help the plant to uptake P from the soil may stay in the soil, even after soil manipulation, being the changes in the soil harmless to plant. In this sense, Wertz et al. (2006) rise the paradigm that soil microbial communities, due to the high diversity, have high functional redundancy levels, and so the erosion of microbial diversity is less important for ecosystem common microbial functions, such as decomposition and respiration. However, these authors demonstrated using the dilution-to-extinction method that the vast diversity of the soil microbiota makes soil ecosystem functioning largely insensitive to biodiversity erosion, even for functions performed by specialized groups. Franklin and Mills (2006), using the dilution-induced reduction in diversity in sewage to study the microbial functional response, observed that the original community was similar to the regrown

community from an inoculum (10^{-6} dilution), and concluded that for each organism type eliminated during the dilution process, at least one of the remaining types was able to provide the same function.

4.4.4 Effect of P application in plant response cultivated under soil with microbiome manipulation

Degraded areas evolve chemical, physical, biological and ecological concepts (Lal 2015), and among the important strategies for soil quality restoration and reducing environmental degradation risks are the improving availability of macro and micro-nutrients.

Our results showed that the P fertilizer application, in general, for all the levels of diversity studied, was not a strong factor to compensate the competition with microorganisms, which did not result in a compensatory effect for shoot dry matter accumulation and shoot P uptake. Oliveira et al. (2001) studied the response to chemical fertilization of degraded pastures in the Brazilian Cerrado, and concludes that N was the most limiting nutrient, compared to P. Besides that, Oliveira et al. (2004) studied chemical and biological indicators of degraded brachiaria pastures in the Brazilian Cerrado and observed that after years of cropping, P concentration in the shoot was not affected. This shows that, even cropping in areas considered degraded, brachiaria does not reduce the P uptake, and so P was not a good indicator of degraded area.

4.5 Conclusion summary

Microbial community was more effective to determine the fate of P during its cycling in the soil, rather than P application. However, plant species showed a strong effect to determine the P fate and the efficiency for P uptake, compared to the microbial community and the P application. Brachiaria and crotalaria are generally used as cover crop and green manure and our results show a potential to use these species in degraded area without loss in plant biomass production. Furthermore, P application did not compensate the high microbial competition effect.

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5 ^{14}C-MINERALIZATION, N TRANFORMATIONS, ^{31}P AND ^{33}P-FRACTIONATION OF SOILS SUBMITTED TO MICROBIOME MANIPULATION BY AUTOCLAVING: A LABORATORY STUDY

Abstract
Soil autoclaving changes the microbial community, what may affect the chemical characteristics in the soil. The objective was to evaluate ^{14}C-mineralization, N forms, ^{31}P and ^{33}P fractionation in a soil hosting distinct microbiome compositions, generated by natural community dilution method. Soils from native forest (~20 years) and a cultivated area (maize) were used, both collected in Bangor, United Kingdom. Two sets of samples were used in a laboratory, with four replications. In the first set, 250 µL of 20 µM ^{14}C-glucose (1.71 kBq), was added per experimental unit, in 5 g of soil. The ^{14}C activity was monitored: 2, 4, 8 and 24 h after application. Then the soil was submitted to five distinct structures of the native microbiome to compose the treatments: i) natural soil (NS); sterilized soil (SS) by autoclaving (121 ºC; 103 kPa) followed by inoculation of dilution (10% v/w) 24 h after autoclaving: ii) SS+10^{-1}; iii) SS+10^{-3}; iv) SS+10^{-6}); and v) SS, without dilution inoculation. After microbial treatments setup, the ^{14}C activity was monitored: 2, 4 and 8 hours, followed by: 1, 2, 3, 4, 5, 7, 10, 14, 18, 23, 28 days after microbiome manipulations. The results showed that, in the SS, $^{14}CO_2$ increaments were null and no changes in total N compared to NS, but in the proportion between NH_4 and NO_3, increasing N-NH_4 in the SS. The inoculations of dilutions SS+10^{-1} and SS+10^{-3} showed rates of $^{14}CO_2$ higher compared to NS for both soils, and an increament in total N, due to increament of N-NH_4. In the second set of samples, 25µL of ^{33}P-water (7.23 kBq), was added per experimental unit, in 1 g of soil. Then the soil was submitted to the same five distinct structures of the native microbiome to compose the treatments, as described. The soil was incubated for 14 days and then ^{31}P and ^{33}P fractionation was performed. The autoclaving procedure, even after inoculation of dilutions, did not change the ^{31}P lability, but reduced the ^{33}P-labile activity and increased the ^{33}P-non-labile activity for both soils. Combined, the results show that the autoclaving procedure reduced the activity of the microorganisms, and hence changed the proportion between N-NH4 and N-NO_3, but not effect on the total N. The inoculation of high number of cells in the autoclaved soil was able to promote changes in the amount of total N, indicating that the changes were promoted by the microbiota and not by the autoclaving itself.

Key words: Diversity extinction; Respiration; Ammonium; Nitrate; P lability

5.1 Introduction

Soil microorganisms play an important role in carbon (C), nitrogen (N) and phosphorus (P) biogeochemical cycling. Regarding the N cycle, the processes of nitrogen fixation, mineralization (decay), nitrification and denitrification are played by microorganisms (Hayatsu et al. 2008). For P, the immobilization within the microbial biomass becomes a temporary P reservoir in the soil, and upon microbial death this P becomes available again. The mineralization of organic P is affected by the size, activity and composition of the microbial

community through the exudation of a range of phosphatase exoenzymes (Kandeler et al. 2002), which may be promoted also by the plant itself. Also, P can be released over the time by acidification processes, through microbial organic acid production and exudation (Li et al. 2008). Therefore, changes in the composition of the microbial community may change the functioning of the soil system.

Under controlled conditions, different methodologies can be used to sterilise the soil to promote or reduce the soil microbial community and diversity, such as ethylene oxide (Clark 1950), anaerobic disinfestation (Mazzola et al. 2018); gamma irradiation (Ogwu et al. 2019). Another method is the autoclaving procedure, which is fast, cheap and the equipment is part of the facilities in most of the microbiology laboratories, and additionally has the advantage of not generating soil contaminated with chemicals (Skipper and Westermann 1973). This technique works with the combination of high temperature and pressure.

In this sense, after autoclaving the soil, microbial activity is reduced mainly due to the loss of most of microorganisms. In the previous chapter of this thesis, changes promoted in the soil by manipulation using autoclaving were detected. We observed in the sequencing analysis, for both fungi and bacteria, based on DNA, that the diversity in the soil submitted to the autoclaving procedure was similar to the natural soil, without much changes in diversity and richness index. Therefore, the techniques based on DNA did not show to be adequate. Apparently, autoclaving is not able to degrade the relic DNA (Carini et al. 2017). In the previous chapter of this thesis we also evaluated the acid phosphatase activity, and despite the reduction of the enzyme activity after autoclaving, it did not reach null levels. According to Carter et al. (2007), besides killing the soil microbes by autoclaving, the soil enzymes remained active, therefore showing a poor correlation between the microbial activity in the soil. Thus, an alternative to evaluate the soil microbial activity after autoclaving is the use of techniques that monitor microbial process, such as soil microbial respiration and microbial C partitioning.

In addition, autoclaving, due to the combination of high temperature and pressure, may induce some chemical changes in the soil, affecting the availability of some nutrients, such as N and P (e.g. through the release of organic acids from lysed microbial cells, thermal hydrolysis of organic matter, changes in Fe chemistry etc), promoting alterations either directly by the autoclaving conditions or indirectly by the loss of microorganisms that participate in the nutrient cycling.

In the view of the above, the objectives of this work were to evaluate the effect of the autoclaving procedure on soil microorganism's activity through the analysis of $^{14}CO_2$, N-NO_3, N-NH_4, ^{31}P and ^{33}P in the soil under contrasting microbial communities.

5.2 Material and methods

5.2.1 Experimental setup

Two soil samples were collected from the Henfaes Experimental Station, Wales, UK. Both soils were classified as Eutric Cambisols (WRB-FAO, 2015) and were collected from the Ah horizon (0-10 cm depth) from under either a 24-year old Sycamore (*Acer pseudoplatanus*) plantation (53°14'22.70" N, 4°0'54.81"W) or an adjacent cultivated area growing maize (*Zea mays* L.; 53°14'19.94"N, 4°0'53.71"W) (Table 1). The agricultural field receives 120 kg N, 60 kg K and 10 kg P y^{-1}, while no fertiliser has historically been applied to the forest plot. Prior to planting, the forest was under the same agricultural regime as the maize plot. After collection, the soil was placed in gas-permeable plastic bags, sieved to pass 5 mm and stored at 4 °C until required. The mean annual temperature at the site is 10 °C and the annual rainfall is 1060 mm.

Table 1. Chemical analysis of the forest and cultivated soil before the experiment.

Treatment	pH	P	K	Mg	Ca	S	Mn	Cu	B	Zn	Mo	Fe	CEC
							mg kg^{-1}						meq $100g^{-1}$
Forest	5.4±0.1	17±0.8	87±1.7	133±2.4	1149±26.6	5.0±0.0	87±8	7.2±0.1	0.74±0.04	7.2±0.2	0.08±0.02	784±46	11.0±0.5
Cultivated	6.4±0.1	36±0.8	74±3.3	70±2.2	1851±96.2	4.0±0.0	94±6	9.6±0.5	0.97±0.05	7.2±0.2	0.08±0.01	854±21	11.3±0.5

Values represent means ± standard error of the mean SEM (n = 4).

5.2.2 ^{14}C-glucose application and manipulation of the soil microbiome

Replicate samples of each field-moist soil (5 g) were placed in individual sterile 50 cm^3 polypropylene centrifuge tubes. Subsequently, 250 µL of 20 µM ^{14}C-glucose (6.84 kBq mL^{-1}) was applied to the soil and the mineralization of the ^{14}C-glucose was determined by capturing any $^{14}CO_2$ evolved in 1 M NaOH traps placed above the soil surface (Glanville et al. 2016). The NaOH traps were replaced after 2, 4, 8 and 24 h after ^{14}C-glucose application. The amount of ^{14}C in the NaOH traps was determined using Optiphase HiSafe 3 scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA) and a Wallac 1404 liquid scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK).

After 24 h, the ^{14}C-soil was subjected to an extinction-dilution methodology to generate five contrasting soil microbiomes with 4 replicates of each treatment. The first treatment consisted of untreated natural soil (NS). This was used as the positive control for all comparisons. In addition, four treatments were obtained by sterilising the soil by autoclaving (121 ºC, 103 kPa, 1 h), followed by re-inoculation according to the dilution-extinction methodology. Briefly, a 10% (v/w) soil suspension was made by adding natural soil to deionized and sterilized water and shaking at 200 rev min^{-1} for 1 h. The sterilised soil (SS) was then re-inoculated using 250 µL the soil suspension at rates of either: i) SS+10^{-1}; ii) SS+10^{-3}; iii) SS+10^{-6}); and iv) SS, without dilution inoculation (van Elsas et al. 2012).

After setting up the five soil microbiome treatments, the $^{14}CO_2$ evolution from the soils was again monitored by changing the NaOH traps after 2, 4 and 8 h, followed by 1, 2, 3, 4, 5, 7, 10, 14, 18, 23 and 28 days after soil microbiome manipulation and determining their ^{14}C activity as described above. After 28 d, the soils were extracted with 25 mL of 0.5 M K_2SO_4 by shaking at 200 rev min^{-1} for 15 min. The extract was then centrifuged (18,000 *g*, 15 min) and ^{14}C in the supernatant measured by liquid scintillation counting as described above. In addition, the amount of N-NO_3 and N-NH_4 in the soil extracts was determined colorimetrically using the methods of Maynard et al. (1993), Miranda et al. (2001) and Mulvaney (1996), respectively.

5.2.3 ^{33}P application and manipulation of the soil microbiome

Replicate samples of each field-moist soil (1 g) were placed in individual sterile 50 cm^3 polypropylene centrifuge tubes. Subsequently, 25 µL of ^{33}P-H_2O (289.2 kBq mL^{-1}) was applied to the soil. After equilibrating the ^{33}P in the soil for 24 h, the soil was submitted to the extinction-dilution methodology as described above to generate five different soil microbiomes, re-inoculated using 50 µL the soil suspension.

Subsequently, the soils were incubated for 14 d and then the soil was subjected to a sequential ^{31}P and ^{33}P fractionation procedure. Briefly, the soil was first extracted (200 rev min^{-1}, 16 h) with 30 mL deionized water in the presence of a capsule containing a mixed cation-anion exchange resin (Unibest International, Kennewick, WA, Australia). After, ^{31}P and ^{33}P were desorbed from the resin by placing the capsule in 30 mL of 0.5 M HCl for 16 h. The soil was then centrifuged (3850 *g*, 30 min) and the water discarded. The remained soil was then extracted with 30 mL of 0.5 M $NaHCO_3$ (200 rev min^{-1}, 16 h), centrifuged (3850 *g*, 30 min) and the supernatant recovered. This process was repeated with the following extractants: 0.1 M NaOH, 1.0 M HCl and 0.5 M NaOH. After all the extractions, the remaining soil was dried, grounded, and 0.1 g of soil was digested with perchloric acid (10 mL, 200 ℃, 4 h). ^{31}P content in the extracts was determined by colorimetrically according to Murphy and Riley (1962). ^{33}P activity was determined by using 1 mL of each extractant with 4 mL of Optiphase HiSafe 3 scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA) and a Wallac 1404 liquid scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK .

5.2.4 Calculation and statistical analysis

The amount of ^{14}C immobilized in the microbial biomass ($^{14}C_i$) was estimated according to:

$$^{14}C_i = {}^{14}C_a - ({}^{14}CO_2 + {}^{14}C_s)$$

where $^{14}C_a$ is the total ^{14}C applied at the begging of the experiment, $^{14}CO_2$ is the total amount of ^{14}C recovered in the NaOH traps, and $^{14}C_s$ is the ^{14}C recovered in the K_2SO_4 0.5 M extracts.

Microbial carbon use efficiency (^{14}CUE) was calculated by the equation:

$$^{14}CUE = {}^{14}C_i / ({}^{14}C_i + {}^{14}CO_2)$$

Due to the relatively rapid decline in ^{33}P activity by radioactive decay (25.34 days) (Audi et al. 2003), all data were decay-corrected back to the start of each experiment, the time point of tracer was added to the soil, according to:

$P_0 = P / e^{-\lambda t}$

where P_0 is the decay-corrected ^{33}P activity in a sample (kBq), P is the measured ^{33}P activity at time of liquid scintillation counting (kBq), t is time (days) elapsed between tracer addition and ^{33}P activity measurement, e = 2.71828 and λ is the decay constant of ^{33}P (0.0273539).

Comparisons among manipulated microbiome treatments were made by one-way analysis of variance (ANOVA) for each forest and cultivated soil separately. Differences between treatments were performed using the Tukey test where $p \leq 0.05$ was used as the cut-off for statistical significance.

5.3 Results

5.3.1 ^{14}C dynamics from soils under microbiome manipulation

The initial rate of $^{14}CO_2$ production in the natural soil (NS) without microbial manipulation was similar in both the forest and cultivated soil (Figure 1). After this initial phase the production of $^{14}CO_2$ stabilised around 30% of the initial ^{14}C-glucose activity applied. When the soil was submitted to sterilization, no $^{14}CO_2$ emission was observed in both soils, forest and cultivated. On the other hand, when the dilutions SS+10^{-1} and SS+10^{-3} were inoculated into the sterile soil, a rapid and high increase in the $^{14}CO_2$ emission was observed in both treatments. However, when the most diluted inoculation (SS+10^{-6}) was employed, increments in $^{14}CO_2$ emission were observed only in the cultivated soil (Figure 1B).

In general, for both soils, forest and cultivated, the trend of the distribution of ^{14}C was similar (Figure 2A, C). The highest ($p \leq 0.05$) $^{14}CO_2$ emissions were observed in the SS+10^{-1} followed by SS+10^{-3} and consequently these treatments showed low ($p \leq 0.05$) ^{14}C-immobilization and ^{14}C remaining in the soil. Intermediary emissions of $^{14}CO_2$ with high ($p \leq 0.05$) immobilization and the lowest ^{14}C remaining in the soil were observed in the NS treatment. The lowest ($p \leq 0.05$) $^{14}CO_2$ emissions, and the highest ^{14}C remaining in the soil were observed under the SS+10^{-6} and SS treatments, but ^{14}C-immobilization was similar to NS.

Regarding to the carbon use efficiency (^{14}CUE), for both forest and cultivated soils, the highest values were observed in the SS, and SS+10^{-6} for the forest soil (Figure 2B, D), followed by the NS. The lowest ($p \leq 0.05$) ^{14}CUE was observed under SS+10^{-1} and SS+10^{-3}, for both forest and cultivated soil.

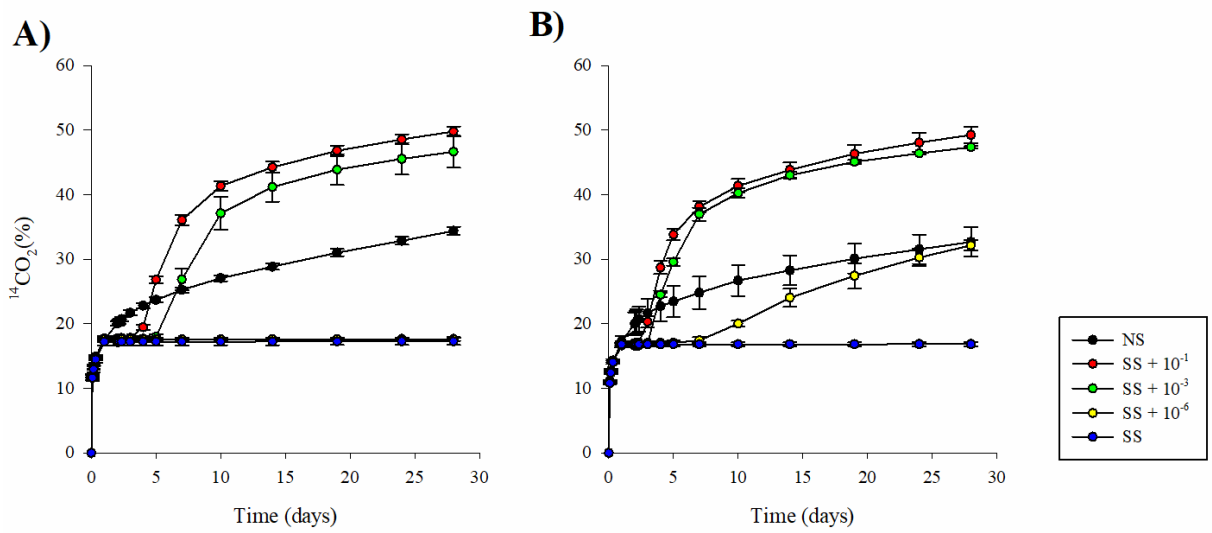

Figure 1. $^{14}CO_2$ emission as a function of time from the agricultural and forest soils: **A)** Forest (24 years old) and **B)** cultivated area (maize), after being subjected to soil microbiome manipulation. Treatment abbreviations are defined as follows: NS: natural soil, without manipulation; SS: sterilized soil by autoclaving (121 ºC, 103 kPa, 1 h), followed by inoculation of dilutions: SS+10^{-1}; SS+10^{-3}; and SS+10^{-6}. Error bars indicate the standard deviation.

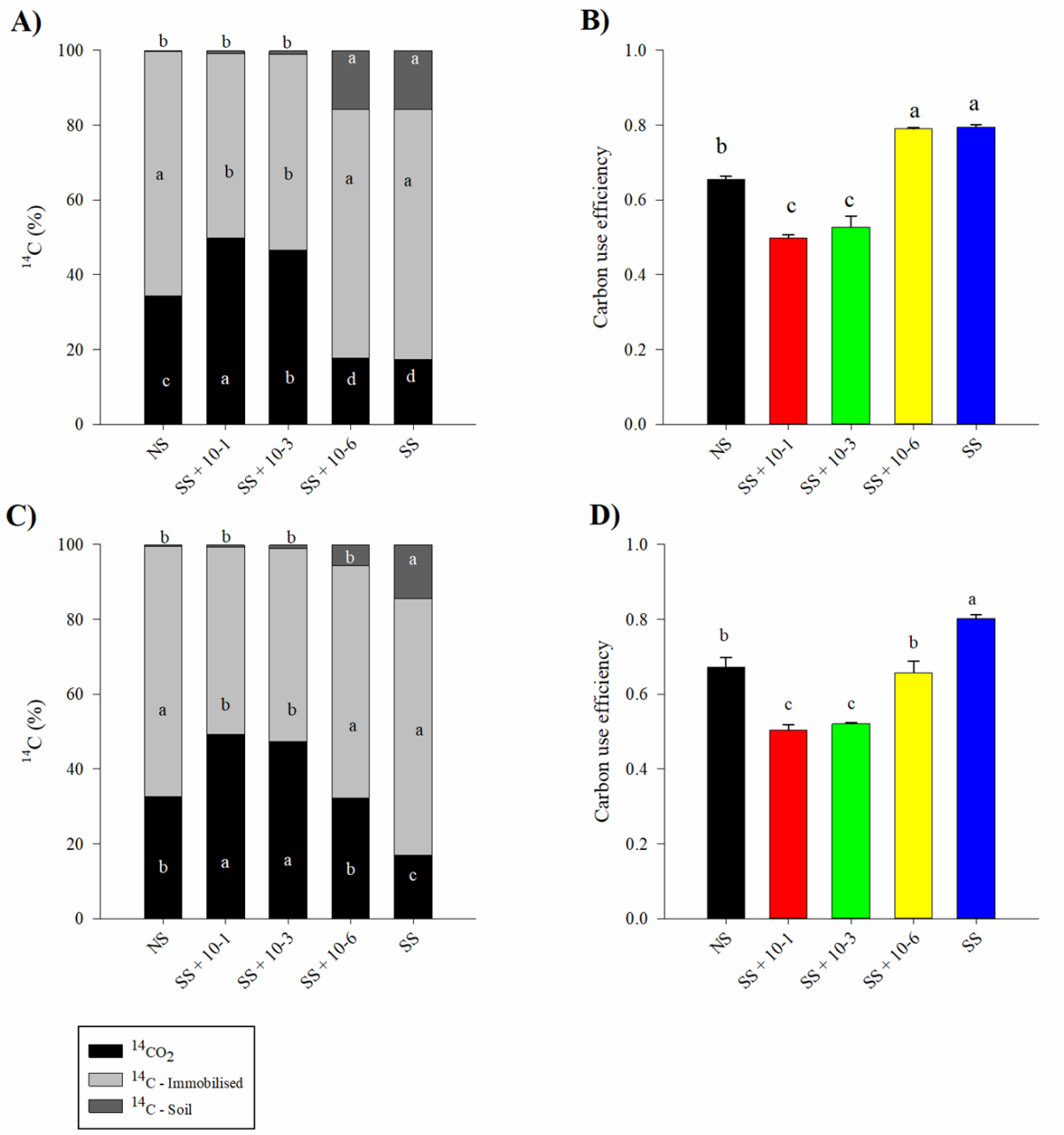

Figure 2. A) and **C)** shows the amount of ^{14}C-glucose which was recovered in $^{14}CO_2$ emission, ^{14}C immobilised in the microbial biomass and ^{14}C remaining in the soil (unused substrate). **B)** and **D)** Carbon use efficiency (%), after 28 days experiment in relation to the ^{14}C applied before the soil microbial manipulation: Treatment abbreviations are defined as follows: NS: natural soil, without manipulation; SS: sterilized soil by autoclaving (121 ºC, 103 kPa, 1 h), followed by inoculation of dilutions: SS+10^{-1}; SS+10^{-3}; and SS+10^{-6}. **A)** and **B)** Forest (24 years old) and **C)** and **D)** cultivated area (maize). Different letters indicate a significant difference between the soil microbial manipulation treatments ($p \leq 0.05$) by Tukey test. Error bars indicate the standard deviation.

5.3.2 N from soils under microbiome manipulation

For both forest and cultivated soils, the total N did not show difference between NS, and SS, and an increment in SS+10^{-6} was observed in cultivated soil,. On the other hand, the highest total N was observed in SS+10^{-1} and a reduction was observed in SS+10^{-3} in forest, but no difference was observed in cultivated soil between SS+10^{-1} and SS+10^{-3} (Figure 3).

Regarding to the forms of N, for the forest, the highest ($p \leq 0.05$) N-NO_3 was observed under NS, without soil microbiome manipulation, and all the other treatments showed lower N-NO_3 but no differences between each other (Figure 3). A similar trend was observed in the cultivated soil, however, both NS and SS+10^{-1} showed the highest values, but no difference between them was observed. The other treatments showed low N-NO_3 (Figure 3). Related to the magnitude, cultivated soil showed lower N-NO_3 values compared to the forest.

Regarding the levels of N-NH_4, in general, for both forest and cultivated soils, the highest value was observed under SS+10^{-1}, followed by SS+10^{-3}, intermediated values were observed under SS+10^{-6} and SS, and the lowest values was observed under NS (Figure 3).

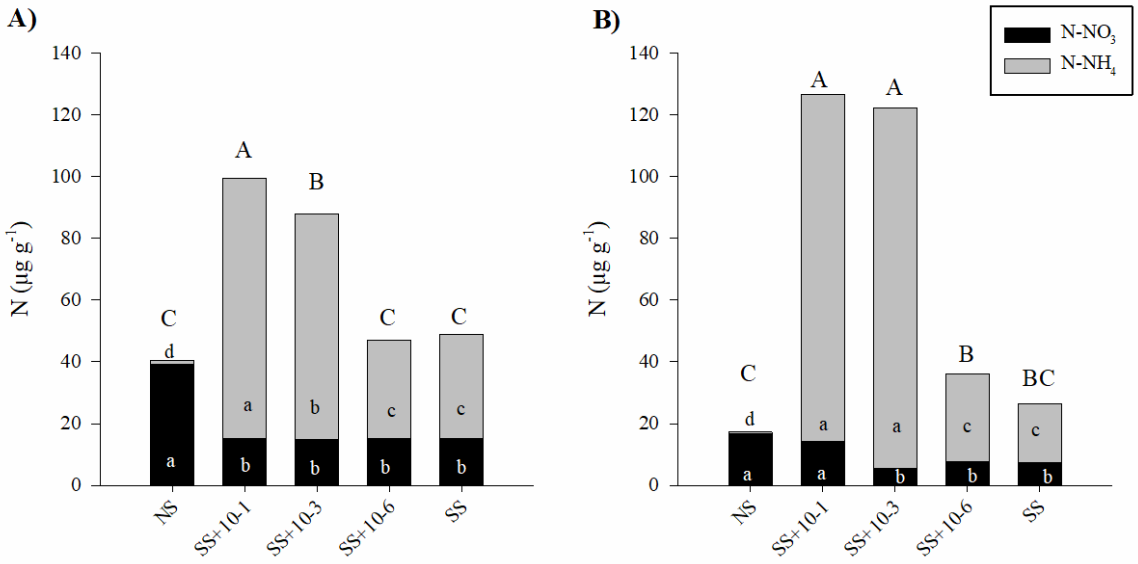

Figure 3. N-NO_3 and N-NH_4 for: **A)** Forest (24 years old) and **B)** cultivated area (maize), after 28 days experiment in relation to the ^{14}C applied before the soil microbial manipulation: Treatment abbreviations are defined as follows: NS: natural soil, without manipulation; SS: sterilized soil by autoclaving (121 ºC, 103 kPa, 1 h), followed by inoculation of dilutions: SS+10^{-1}; SS+10^{-3}; and SS+10^{-6}. Different letters indicate a significant difference between the soil microbial manipulation treatments regarding to N-NO_3 and N-NH_4 ($p \leq 0.05$) by Tukey test. Error bars indicate the standard deviation.

5.3.3 ^{31}P and ^{33}P fractionation from soils under microbiome manipulation

In general, no difference was observed for all the ^{31}P fractions according to the lability for both forest and cultivated soil (Figure 4A). On the other hand, ^{33}P activity in all fractions, for both forest and cultivated soils, showed an effect of microbiome manipulation, except for the $^{33}P_{NaHCO3}$ fraction under cultivated soil (Figure 4B).

In general, the trends between the forest and the cultivated soils were similar for the ^{33}P activity for the labilities. For the labile fractions, the highest $^{33}P_{Resin}$ activity was observed under NS, and no difference between the other treatments was observed, which was observed in the forest for $^{33}P_{NaHCO3.}$ For the moderately ($P_{0.1MNaOH}$ and P_{HCl}) and non-labile fraction ($P_{0.5M\ NaOH}$ and $P_{Residual}$), for both forest and cultivated soil, the lowest ^{33}P activity was observed under NS, and in general, no difference was observed between the other treatments.

Regarding the recovery of ^{33}P in relation to the initial ^{33}P activity, for the forest, no effect of the soil microbiome manipulation was observed, and on average 86% of the ^{33}P was recovered by the fractionation procedure. On the other hand, for the cultivated soil, the highest recovery values were observed under manipulation of the soil microbiome and the lowest recovery was observed under NS (data not shown).

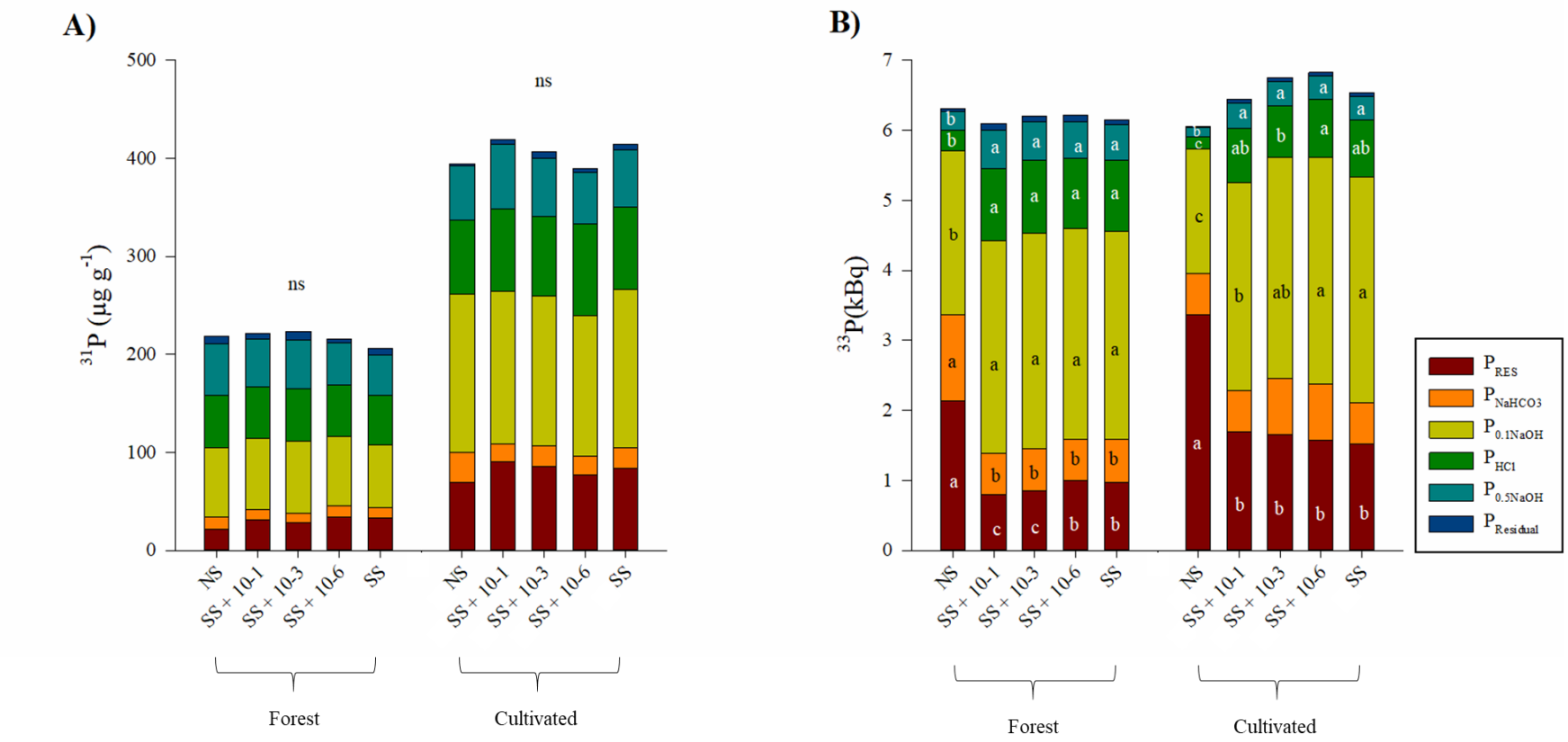

Figure 4. A) P fractionation and **B)** ^{33}P fractionation according to the ^{33}P extractants: resin (P_{RES}); 0.5 M $NaHCO_3$ (P_{NaHCO3}); 0.1 M NaOH ($P_{0.1NaOH}$); 1.0 M HCl (P_{HCl}); 0.5 M NaOH ($P_{0.5NaOH}$); and digestion with perchloric acid ($P_{Residual}$), for forest soil (24 years old) and cultivated area (maize), after 28 days experiment in relation to the ^{14}C applied before the soil microbial manipulation: Treatment abbreviations are defined as follows: NS: natural soil, without manipulation; SS: sterilized soil by autoclaving (121 ºC, 103 kPa, 1 h), followed by inoculation of dilutions: SS+10^{-1}; SS+10^{-3}; and SS+10^{-6}.Different letters indicate a significant difference between the soil microbial manipulation treatments. ns: no significant ($p > 0.05$).

5.4 Discussion

In general, our results showed that both forest and cultivated soils presented the same trend after autoclaving treatment. The autoclaving procedure has the objective to sterilise the soil, promoting the death of most of the organisms and consequently loss of them, creating niches, by favouring some processes to the detriment of others, and so promoting the reshaping the microbial community (Li et al. 2019).

In general, regarding the microbial activity, NS differed from SS+10^{-6}, with inoculation of low number of cells, and SS, autoclaved treatment and non-inoculation (SS). After ^{14}C-glucose application and the autoclaving procedure, the soil microorganisms under SS and SS+10^{-6} did not showed emission of $^{14}CO_2$ compared to the NS, indicating low activity, even after a ^{14}C easy degradable source application, as glucose. Cellulose is the most abundant biopolymer in a plant-soil system (Kögel-Knabner 2002), and its decomposition become sub products, such as glucose, bioavailable to the microorganisms. Furthermore, glucose is the most product released in the rhizosphere (Derrien et al. 2004), characterizing this environment. Therefore, the addition of glucose is a logical tool to understand the priming effect in the soil (Kuzyakov 2010), and so correlate with the microbial activity.

However, few similarities between NS, SS+10^{-6} and SS were observed. The ^{14}C immobilized, total N and ^{31}P lability did not change after autoclaving, indicating that the procedure was not able to change those chemical attributes of both soils.

Considering the immobilized ^{14}C, formed from the easily degradable ^{14}C-glucose, NS, SS+10^{-6} and SS showed similar results, which indicated that the difference among these treatments was the microbial activity and so the loss of $^{14}CO_2$, but not the ^{14}C immobilized.

Another similar result among NS, SS+10^{-6} and SS was the total N. Even though no changes in total N were observed, the forms of the N changed after autoclaving. In NS, a predominance of N-NO_3 is observed, and a little amount of N-NH_4. After autoclaving, the opposite dynamics was observed, a decrease in N-NO_3 and the accumulation of N-NH_4 counted for most of the total N. During the mineralization of the organic matter, the decomposers release ammonium (N-NH_4) to the soil. Serrasolsas and Khanna (1995) studied the changes in heated and autoclaved forest Australian soils and observed an increment in N-NH_4 under the heating (120°C) to soil, and theese authors related these results to the death of the microorganisms, and mineralization of the soil organic N, which was also observed by Giovannini et al. (1990).

From $N\text{-}NH_4$, nitrifiers produce nitrite ($N\text{-}NO_2$), and from this the nitrobacter can produce nitrate ($N\text{-}NO_3$). Our results indicated that after autoclaving the nitration and nitrosation are not occurring in the soil, which may be due a loss of specialized organisms and part of the $N\text{-}NO_3$ was converted in $N\text{-}NH_4$. Domsch et al. (1983) used an ecological concept to study the soil microbial parameter and classified nitrifiers as high sensitive to perturbations.

Furthermore, ^{33}P fractionation indicated that the autoclaving procedure reduced the labile ^{33}P compared to the NS, ^{31}P lability did not change. Considering the changes in ^{33}P lability, the inoculation of the dilutions in the autoclaved soil, in general, did not alter the lability, compared to SS. This indicates that the changes were due to the autoclaving, but not by the microorganisms. However, these changes were not detected in ^{31}P, indicating that the buffer of the soil stabilized the lability changes promoted by the autoclaving.

When the soil was autoclaved and inoculated with dilutions 10^{-1} ($SS+10^{-1}$) and 10^{-3} ($SS+10^{-3}$), considering high number of organisms reinoculated, high $^{14}CO_2$ emission was observed. The high number of microorganisms inoculated after ^{14}C-glucose application and autoclaving found an environment very rich in ^{14}C-glucose, that triggering the apparent priming effect to the microorganisms. Our results show a rapid increase of $^{14}CO_2$, indicating a high decomposition in $SS+10^{-1}$ and $SS+10^{-3}$ during the first two weeks, corroborating with Pascault et al. (2013). These authors stimulated the soil priming effect of different functional groups of bacteria by using various plant residues, and attributed this rapid initial consuming in the first two weeks to the presence of easily degradable compounds. In our case, glucose, easily degraded, was rapidly used by the microorganisms inoculated under $SS+10^{-1}$ and $SS+10^{-3}$. This rapid usage of ^{14}C is performed by the r-strategist, or copiotrophs. Once the C source is over, these organisms died or become dormant (Bernard et al. 2007; Fontaine et al. 2003), and occur the establishment of the basal activity, then we see a stabilization in $^{14}CO_2$ emission.

Considering the N cycling, after inoculation of the dilution $SS+10^{-1}$ and $SS+10^{-3}$, a huge accumulation of $N\text{-}NH_4$ is observed. Besides the transformation from $N\text{-}NH_4$ to $N\text{-}NO_3$ is not occurring, which may be due the loss of specialised group responsible to the nitration and nitrosation after autoclaving, we suggest an increment in the mineralization of organic matter, either by the high number of cells, or by the high microbial activity that promotes the mineralization of the organic matter present in the soil is being breaking down and releasing $N\text{-}NH_4$ in the soil.

Therefore, the N transformations were increased by the inoculation of the dilutions 10^{-1} and 10^{-3}, which indicates that the microbial activity was more effective to change the N, rather than the autoclaving itself. Silver et al. (2001) showed that in a sterile soil the dissimilatory reductive pathway from both NO_2 and N-NO_3 to N-NH_4 was predominantly biotic, and related this process to favour the assimilation of NH_4 by plants and microbes, because NO_3 is easily lost from the ecosystem, being this transformation an important role in ecosystem N conservation.

To compare the decomposers C metabolisms among the treatments, we used the carbon use efficiency (CUE). The highest values of ^{14}CUE were observed under SS and SS+10^{-6}, due to the low release of $^{14}CO_2$ and so high accumulation in the biomass, while the opposite was observed under SS+10^{-1} and SS+10^{-3}, where high $^{14}CO_2$ was lost to atmosphere and less ^{14}C was converted to biomass (Manzoni et al. 2012).

5.5 Conclusion summary

Our results of $^{14}CO_2$ emission indicated that the autoclaving procedure reduced the activity of the microorganisms, and hence changed the proportion between N-NH_4 and N-NO_3, but not effect on the total N.

The inoculation of high number of cells in the autoclaved soil was able to promote changes in the amount of total N, indicating that the changes were promoted by the microbiota and not by the autoclaving itself.

The autoclaving procedure, even after inoculation of dilutions, did not change the ^{31}P lability, but reduced the ^{33}P-labile activity and increased the ^{33}P-non-labile activity for both soils.

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6 BRACHIARIA AND CROTALARIA CULTIVATED IN TWO SOILS FROM UNITED KINGDOM WITH DISTINCT MANIPULATED MICROBIOME UNDER ^{33}P-PHOSPHATE APPLICATION

Abstract
Changes in the microbial community affect the N and P dynamics in the soil. The objective of this study was to evaluate the N-NH_4, N-NO_3 and PO_3^- dynamics in the soil and plant N and P uptake cultivated in soil hosting distinct microbiome communities under P-^{33}P application along two crop cycling. The experiment was conducted in a glasshouse, with four replications. Two soils were used: i) forest soil; ii) cultivated soil. The soils used were submitted to two distinct manipulated microbiome communities: *i)* natural soil (NS); and *ii)* sterilized soil (SS) by autoclaving (121 ºC; 103 kPa) followed by inoculation of dilution SS+10^{-3}. After the community stabilization, a soil sample was made, and bacteria 16S rDNA sequencing was procedure. At this point, seedlings of brachiaria (*Urochloa brizantha* cv. Marandu) or crotalaria (*Crotalaria juncea* L.) were transplanted to the soils and ^{33}P was supplied: i) without P (^{33}P-water solution; activity = 32 kBq pot^{-1}); and ii) with P application (^{33}P- KH_2PO_4 solution; activity = 32 kBq pot^{-1}; PO_4 = 60 mg kg^{-1}). For 15 weeks, soil solution was sampled and N-NH_4, N-NO_3 and PO_3^- were determined. Two plant cuts were made: 60 and 116 d after transplantation to determine the shoot dry matter, ^{31}P, ^{33}P,and N uptake. Bachiaria showed higher shoot dry matter, P and N uptake under SS+10^{-3} during the 1st cycle. For the 2nd cycle brachiaria, under NS, showed higher N uptake, but no difference in shoot dry matter was observed compared to SS+10^{-3}. Crotalaria was not affected, in both 1st and 2nd cycle, by the microbiome manipulation for shoot dry matter, N and P uptake. Our results indicate that a conversion from either degraded forest area or degraded cultivated area in a brachiaria grass pasture is promising showing superiority in the growth. Crotalaria, in terms of plant growth was not sensible to the changes in the microbial community, and so it is easily adapted for any condition, bringing the advantage of N cycling for the systems.

Key words: nutrient uptake, bacterial composition, P cycling, nitrogen

6.1 Introduction

Soil microorganisms are the principal players in most essential soil functions such as nutrient cycling, pollutant attenuation, carbon storage, water purification, plant supply, etc, but the composition and functioning of the microbial community is modulated by biological interactions and environmental conditions.

Therefore, changes in the microbial community may affect plant-soil interactions and the ability of soils to deliver a range of ecosystem services, but also may lead to a reduction of pathogenic organisms or reduction in the competition between microorganisms and plants.

Many factors may promote changes in the soil microbial community such as the soil management. Forest ecosystems exhibit a high level of spatial heterogeneity and the importance

of trees and microorganisms inhabit various forest habitats, such as foliage, the wood of living trees, litter, soil, deadwood, etc (Baldrian 2017).

The conversion of native areas into cultivated lands will influence the composition, abundance and function of soil microbiome due to changes such as crop cultivation, soil amendments, such as pesticides and fertilizers applications as a source of nutrients, as N, P and K for the plants (Głodowska and Wozniak 2019). The application of fertilization itself may shift the microbial community structures, which could suggest that the positive effect of a single phosphate application on plant growth in a soil can be cancelled out by its negative effect on the soil microbiota and their ecosystem services (Ikoyi et al. 2018).

The hypothesis of this study is that the microbial community, naturally different from forest and cultivated soil, and the derivate communities obtained after a soil manipulation, will generate distinct microbial structures, that will affect the nutrient cycling and further uptake by two crops. Aligned to this hypothesis, the objective was to evaluate the N and P uptake of a legume and a grass under different microbial communities.

6.2 Material and methods

6.2.1 Experimental setup

Two soil samples were collected from the Henfaes Experimental Station, Wales, UK. Both soils were classified as Eutric Cambisols (WRB-FAO, 2015) and were collected from the Ah horizon (0-10 cm depth) from both a 24-year old Sycamore (*Acer pseudoplatanus*) plantation (53°14'22.70" N, 4°0'54.81"W) and an adjacent cultivated area growing maize (*Zea mays* L.; 53°14'19.94"N, 4°0'53.71"W) (Table 1). The agricultural field receives 120 kg N, 60 kg K and 10 kg P y^{-1}, while no fertiliser has historically been applied to the forest plot. Prior to planting, the forest was under the same agricultural regime as the maize plot. The mean annual temperature at the site is 10 °C and the annual rainfall is 1060 mm. After sampling, the soils were placed in gas-permeable plastic bags, sieved to pass 5 mm and stored at 4 °C until required.

6.2.2 Manipulation of soil microbiome

Replicate samples from each field-moist soil (500 g in dry basis) were placed in individual pots and the experiment was conducted in a glasshouse (30 ℃ ± 5; 10 h photoperiod) in randomized block design with four replicates.

In a period of 24 h after sampling, the soil was subjected to an extinction-dilution methodology to generate two contrasting soil microbiomes from each soil. The fresh natural soil (NS) was used as positive control for comparisons in both forest (FOR) and cultivated (CUL) soils. In addition, one treatment was obtained by autoclaving (121 ℃, 103 kPa, 1 h) the soil to obtain sterile samples (SS) for each soil. For 30 d, the moisture of all the pots was maintained in 80% of maximum water holding capacity (WHC) (460 mL kg^{-1}) with deionized and sterilized water to preserve the microbiomes in the soil. Then, the SS were subsequently re-inoculated (30 mL pot^{-1}) using the dilution-to-extinction method. This approach generated the treatment by using the dilution 10^{-3} (v/w of natural soil dilution added to the sterile soil) (van Elsas et al. 2012).

After the setting up of the microbial communities, the soil moisture was maintained at 80% of maximum WHC using deionized and sterilized water during 20 d, when the soil was sampled from all treatments (forest NS; forest SS+10^{-3}; cultivated NS and cultivated SS+10^{-3}). The soil samples were used for chemical analysis: macronutrients (P; K; Mg; Ca; S; NO_3^-; and NH_4^+); micronutrients (Mn; Cu; B; Zn; Mo; Fe; Na) acidity feature (pH), organic matter (OM), and bacterial 16S rDNA sequencing. For chemical features statistical analysis was made by comparison of microbiome using analysis of variance (ANOVA) considering significance ($p \leq 0.05$) by F values, for each soil, forest and cultivated, analysed separately.

6.2.3 Total DNA extraction from soil and 16S sequencing

Total DNA was extracted from soil samples by using the PowerSoil DNA isolation kit (MoBio, Carlsbad, EUA) according to the manufacturer's instructions. Metabarcoding libraries of 16S rDNA amplicons corresponding to V3-V4 variable region were prepared by single PCR with double-indexed fusion primers as described by Fadrosh et al. (2014). 16S rDNA annealing part of forward primers corresponded to forward primer correspond to modified F515 (5′-CACGGTCGKCGGCGCCATT-3′); reverse primer corresponded to modified R806 prokaryotic primer (GGACTACHVGGGTWTCTAAT) (Kublanov et al. 2009). PCR amplification of 16S

rRNA genes was performed by qPCRmix-HS™ SYBR mastermix (Evrogen, Russia) using the following conditions: 30 cycles of denaturation at 95 °C for 15 s; primer annealing at 58 °C, 15 s; DNA synthesis at 72 °C, 25 s, followed by final incubation for 5 min at 72 °C. Purification of PCR products was done using the QIAEX II Gel Extraction Kit (Qiagen). The quality of the final libraries was assessed using the electrophoresis in agarose gel. Libraries were sequenced with MiSeq™ Personal Sequencing System technology of Illumina Inc. (San Diego, CA, USA) using paired-end 250-bp reads. Reads were first trimmed using fastx toolkit and tagcleaner to remove barcodes and adapters from reads. Then, demultiplexing, OTU picking and taxonomic assignation is developed using QIIME 1.9.1 and SILVA 1.3.2 as reference database. Principal Component Analysis (PCA) was made by using soil chemical features.

6.2.4 Pre-germination and seedling of plants

Brachiaria (*Urochloa brizantha* cv. Marandu) and Crotalaria (*Crotalaria juncea* L.) seeds were disinfected by immersing them in 2% (v/v) NaClO solution (15 min), and then rising twice in sterile deionized water. The seeds were sown in sterile and P-free quartz sand. The seedlings were watered with sterile deionized water until being transplanted into the different soil treatment. The pre-seedlings plants, 7 d old, were immersed in deionized and sterilized water for 30 min to facilitate the removal of individuals from the sand, with transplantation occurring 20 d after the microbiome setup.

6.2.5 P, ^{33}P, NO_3^- and NH_4^+ application

One day after plant transplant, for both forest and cultivated soil, two treatments were obtained regarding P supply: i) without P application, where 20 mL pot^{-1} of a ^{33}P-water solution (^{33}P activity = 32 kBq pot^{-1}) was applied (ca. PO_4 <0.001 mg kg^{-1}); and ii) with P application, where 20 mL pot^{-1} of a ^{33}P-labelled KH_2PO_4 solution (^{33}P activity = 32 kBq pot^{-1}; PO_4 = 60 mg kg^{-1}) was applied. At 27 d after plant transplantation, a solution (20 mL pot^{-1}) of KNO_3^- (N = 30 mg kg^{-1}) and $(NH_4)_2SO_4$ (N=56 mg kg^{-1}) was applied in all treatments.

6.2.6 Soil solution sampling and analysis

After plant transplant, soil solution samples were taken weekly using Rhizon® soil solution samplers, for 15 weeks: 30, 37, 44, 51, 58, 65, 72, 79, 86, 93, 100, 107, 114, 120, and

128 d after transplant. One day before the soil solution sampling, the water was monitored and standardized to 80% of maximum WHC in all the pots. In the soil solution we determined: N-NO_3 (Maynard et al. 1993; Miranda et al. 2001); N-NH_4 (Mulvaney 1996); PO_4^- (Murphy and Riley 1962); and ^{33}P activity, by using 1 mL of soil solution, vortexed with 4 mL of Optiphase HiSafe 31 scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA), and the and the ^{33}P activity was counted in a Wallac 1404 liquid scintillation counter (Wallac EG&G, MiltonKeynes, UK).

6.2.7 Harvest of plants and soils sampling

Two cycles of cultivation were conducted for both brachiaria and crotalaria. The first cut was undertaken 60 d after transplanting. The shoot was dried in the over (80°C; 16 h) to obtain the dry biomass. Then, the dried shoot biomass was milled in ball mill to homogenise the sample, to determine N, ^{31}P and ^{33}P uptake. To determine ^{31}P and ^{33}P uptake, 100 mg of the dried biomass was placed into a glass vial and ashed in a muffle (650°C; overnight). Then, 1 mL of 0.5 M HCl was added to the ash and left overnight. An aliquot (100 µL) was taken for ^{31}P determination by colorimetric assay (Murphy and Riley 1962). To determine ^{33}P activity, 900 µL of the solution was vortexed with 10 mL of Optiphase HiSafe 3 scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA), and the ^{33}P activity was counted in a Wallac 1404 liquid scintillation counter (Wallac EG&G, MiltonKeynes, UK).

Brachiaria was allowed to regrow after the first cut and new seedlings of crotalaria were transplanted again into the pots 15 d after the first cut. At the second harvest, 56 d after the first cut, for both brachiaria and crotalaria, the procedure was performed as described in the first cut to determine shoot dry matter, N, ^{31}P and ^{33}P shoot uptake. Plant features statistical analysis was made by comparison of the microbiome and the rates of P using analysis of variance (ANOVA) considering significance ($p \leq 0.05$) by F values. Comparison among soil manipulation treatment and P application were performed using the LSD test ($p \leq 0.05$). The analysis was made separately for the soil, crop specie (brachiaria and crotalaria) and crop cycling (1st and 2nd harvest).

After the second harvest, soil samples were taken and kept (4 ºC) for analysis of: N-NO_3; N-NH_4; PO_4^-; and ^{33}P activity; and (-20°C) for total DNA extraction from soil and 16S rDNA sequencing. To determine N-NO_3 and N-NH_4 an extraction with 25 mL of 0.5 M K_2SO_4 in 5 g of soil was made by shaking at 200 rev min^{-1} for 15 min. The extract was then centrifuged (18,000

g, 15 min) and the amount of NO_3^- and NH_4^+ in the soil extracts was determined colorimetrically using the methods of Maynard et al. (1993), Miranda et al. (2001) and Mulvaney (1996), respectively. To determine ^{31}P and ^{33}P activity, an extraction with 25 mL of 0.5 M CH_3COOH in 5 g of soil was made by shaking at 200 rev min^{-1} for 30 min. The extract was then centrifuged (18,000 *g*, 15 min). The amount of ^{31}P in the soil extracts was determined colorimetrically using the methods of Murphy and Riley (1962), and the ^{33}P activity was determined using 1 mL of the extract vortexed with 4 mL of Optiphase HiSafe 3 scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA), as described above.

Due to the relatively rapid decline in ^{33}P activity by radioactive decay (25.34 days) (Audi et al. 2003), all data were decay-corrected back to the start of each experiment, the time point of tracer was added to the soil, according to:

$$P_0 = P / e^{-\lambda t}$$

where P_0 is the decay-corrected ^{33}P activity in a sample (kBq), P is the measured ^{33}P activity at time of liquid scintillation counting (kBq), t is time (days) elapsed between tracer addition and ^{33}P activity measurement, $e = 2.71828$ and λ is the decay constant of ^{33}P (0.0273539).

Soil features statistical analysis was made by comparison of the microbiome and the rates of P using analysis of variance (ANOVA) considering significance ($p \leq 0.05$) by F values. Comparison among soil manipulation treatment and P application were performed using the LSD test ($p \leq 0.05$). The analysis was made separately for the soil and crop.

Total DNA was extracted from soil samples by using the PowerSoil DNA isolation kit (MoBio, Carlsbad, EUA) according to the manufacturer's instructions. The sequencing was performed as described previously. OTU richness and Shannon (H') index statistical analysis was made by comparison of the microbiome and the rates of P using analysis of variance (ANOVA) considering significance ($p \leq 0.05$) by F values. Comparison among soil manipulation treatment and P application were performed using the LSD test ($p \leq 0.05$). The analysis was made separately for the soil and crop.

6.3 Results

6.3.1 Manipulation of the soil: chemical and bacterial composition shifts

In general, the autoclaving procedure followed by the inoculation 10^{-3} changed most of the micro and macronutrients for both soils, however, the patterns were not the same. For the forest, no changes in Mg, B, Zn and Fe were observed; and for the cultivated area, no differences were observed for Ca and Mo. In both soils, a substantial change was observed in N-NO_3 and N-NH_4. Higher level of NO_3^- was observed NS compared to the SS+10^{-3}. On the other hand, the NH_4^+ in the NS was lower in comparison to the SS+10^{-3} (Table 1).

Considering the microbial community, the bacterial composition,for both forest and cultivated soil, the SS+10^{-3} increased β-proteobacteria (Oxalobacteracea), bacterioidetes and firmicutes, and reduced or eliminated acidobacteria (Figure 1).

Taking together, combining the chemical and microbial shifts, the PCA analysis showed that the bacterial community, both forest and cultivated soil, and microbial manipulation, NSl and SS+10^{-3}, formed four opposite groups, with 82% of explanation. Forest soil was characterized by higher concentration of Mg, while cultivated soil was more relates to high P, Ca, Cu, B, CEC and pH. Regarding to the manipulation, NS soils show the highest figures on Fe, Mo and N-NO_3 and the SS+10^{-3} treatment was more related to high K, S, N-NH_4, Mn, Zn and Na. (Figure 2).

Table 1. Chemical analysis of the forest (24 years old) soil and cultivated (maize) soil used in the experiment. Values represent means ± standard error of the mean (n = 4).

Treatment	pH	Total N	$N\text{-}NO_3$	$N\text{-}NH_4$	P	K	Mg	Ca	S	Mn	Cu	B	Zn	Mo	Fe	CEC
							mg kg^{-1}									$M_{eq}.100g^{-1}$
Forest																
NS	5.4 ± 0.1	68.7 ± 0.9	66.2 ± 1.6	2.5 ± 0.8	17 ± 0.8	87 ± 1.7	133 ± 2.4	1149 ± 26.6	5.0 ± 0.0	87 ± 8	7.2 ± 0.1	0.74 ± 0.04	7.2 ± 0.2	0.08 ± 0.02	784 ± 46	11.0 ± 0.5
SS+10^{-3}	5.7 ± 0.1 *	117.5 ± 17.6 *	3.6 ± 1.0 *	113.9 ± 17.9 *	23 ± 0.5 *	99 ± 1.3 *	129 ± 0.0 ns	1091 ± 3.3 *	8.8 ± 0.5 *	238 ± 14 *	6.3 ± 0.2 *	0.76 ± 0.03 ns	7.2 ± 0.2 ns	0.03 ± 0.02 *	680 ± 29 ns	9.8 ± 0.7 *
Cultivated																
NS	6.4 ± 0.1	27.8 ± 4.3	26.9 ± 4.1	0.9 ± 0.03	36 ± 0.8	74 ± 3.3	70 ± 2.2	1851 ± 96.2	4.0 ± 0.0	94 ± 6	9.6 ± 0.5	0.97 ± 0.05	7.2 ± 0.2	0.08 ± 0.01	854± 21	11.3 ± 0.5
SS+10^{-3}	6.8 ± 0.1 *	117.2 ± 22.5 *	1.6 ± 0.7 *	115.6 ± 22.5 *	41 ± 1.0 *	132 ± 7.5 *	74 ± 2.6 *	1846 ± 64.8 ns	8.3 ± 0.5 *	188 ± 17 *	8.8 ± 0.5 *	1.04 ± 0.02 *	8.1 ± 0.4 *	0.05 ± 0.01 ns	656 ± 56 *	12.4 ± 0.3 *

Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3}. * Significant ($p \leq 0.05$) by F test; ns: non-significant ($p > 0.05$) by F test.

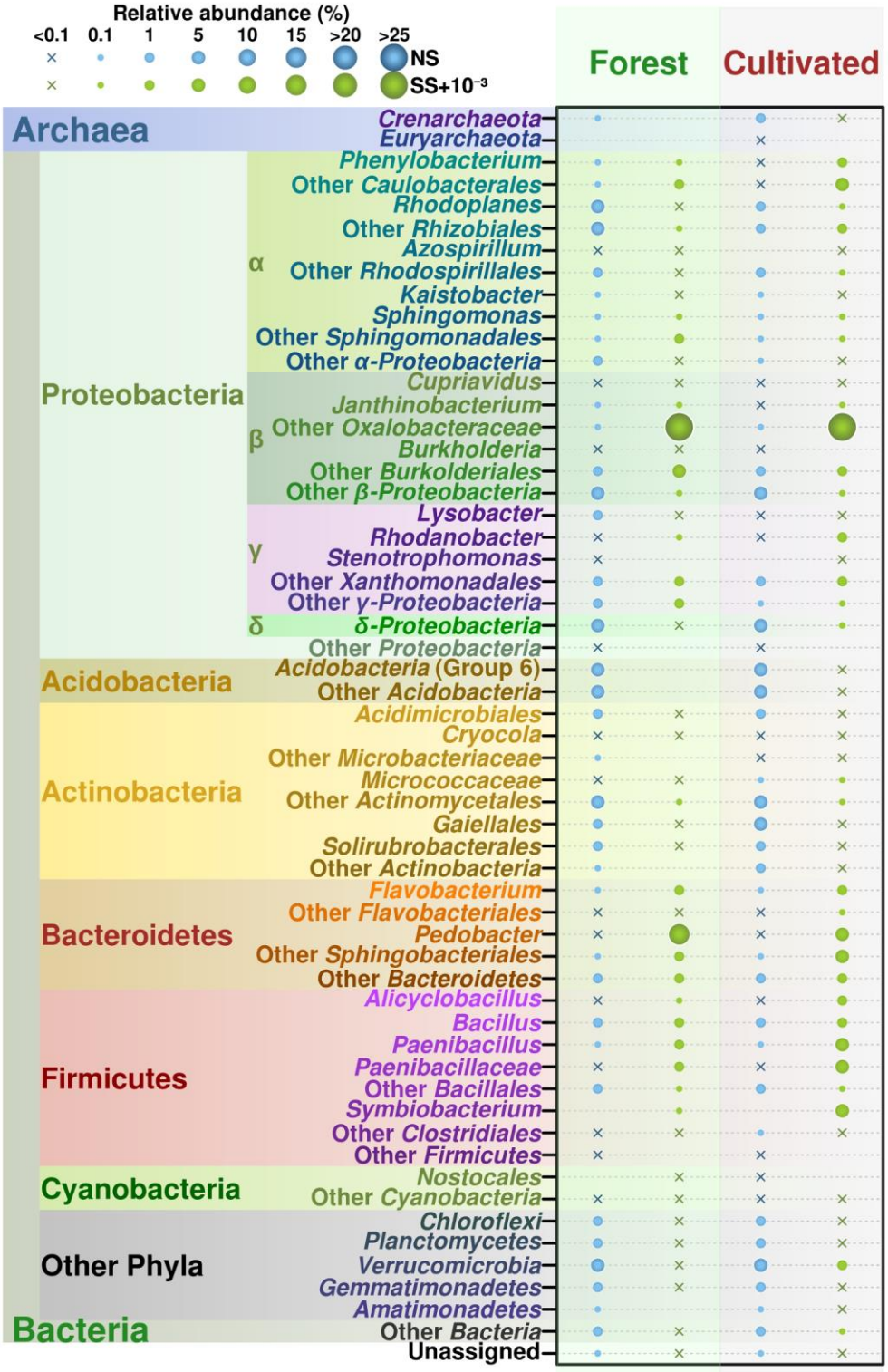

Figure 1. Relative abundande for bacteria community. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions (SS+10^{-3}). Forest (24 years old) soil and cultivated (maize) soil used in the experiment.

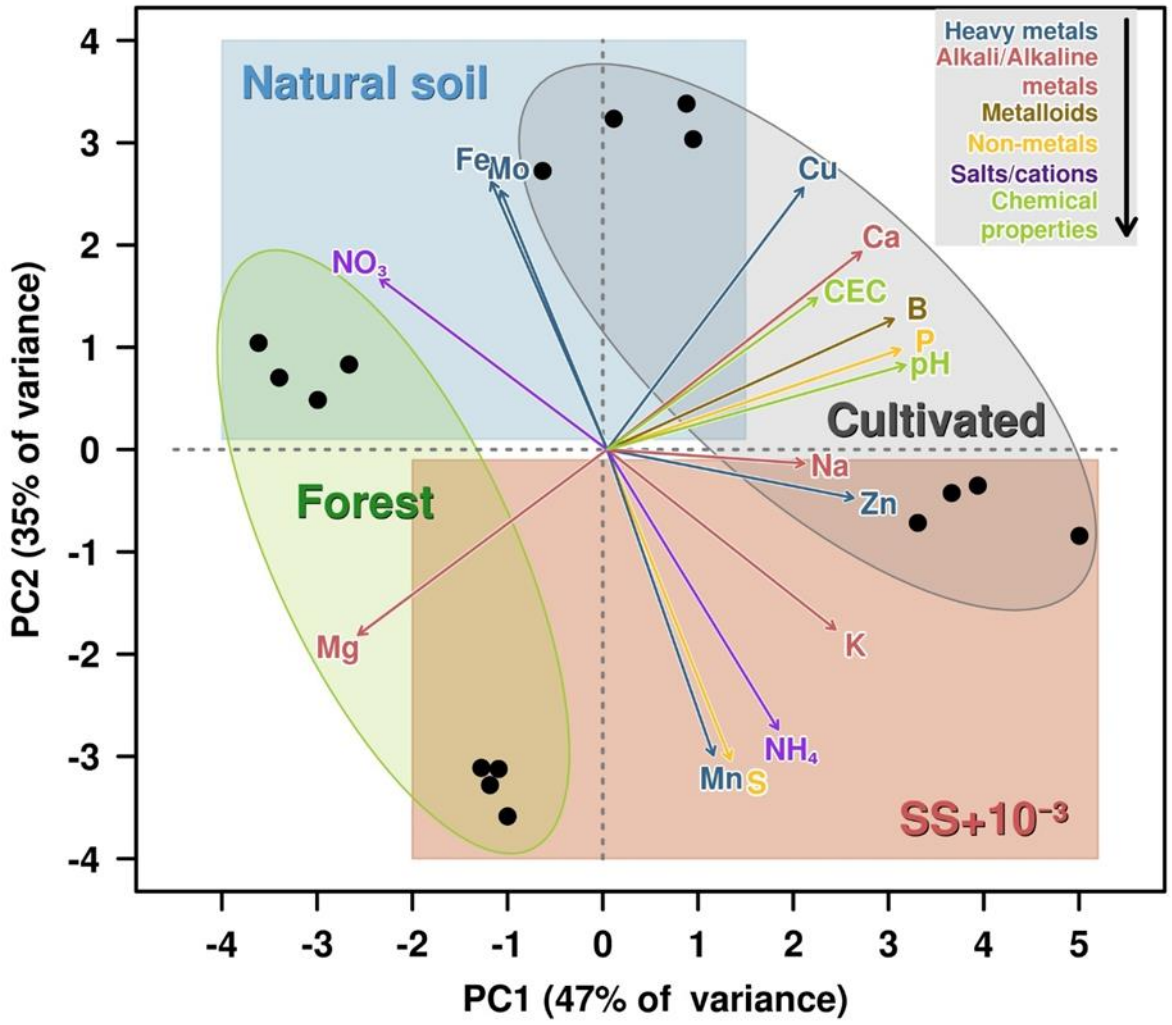

Figure 2. Principal component analysis (PCA) biplot of bacteria community on the soil chemical variables (arrows). Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions (SS+10^{-3}). Forest (24 years old) soil and cultivated (maize) soil used in the experiment.

6.3.2 Effect of brachiaria and crotalaria in the soil solution during cultivation under manipulated soil

N-NH_4 in the soil solution, for both brachiaria and crotalaria, cultivated under FOR and CUL, was affected by the manipulation of microbiome treatments, but not for the P application, where the highest levels, 30 days after transplanting, were observed under SS+10^{-3}, compared to the NS, and FOR showed higher values of N-NH_4 in the soil solution compared to the CUL (Figure 3). For brachiaria, the levels of N-NH_4 in the soil solution dropped to very low levels for all the treatments, in the FOR (Figure 3A) and in the CUL

(Figure 3B), few days before the 1st harvest, that occurred 60 days after transplanting, and no increments were observed until the 2nd harvest for all the treatments. For crotalaria, under SS+10^{-3} in FOR, the high level of N-NH_4 in the solution was maintained even after the 1st harvest, but dropped during the 2nd cycle to low values until the 2nd harvest (Figure 3C). NS, for both FOR and CUL, cultivated with brachiaria and crotalaria, showed low levels of N-NH_4 in the soil solution from 30 days after transplanting and were maintained until the 2nd harvest (Figure 3).

The levels of N-NO_3, for both brachiaria and crotalaria, at 30 days after transplanting were higher under NS, compared to the SS+10^{-3}. For brachiaria, both FOR and CUL, the levels of N-NO_3 in the soil solution, were reducing across the 1st cycle and after the 1st harvest the levels were very low until the 2nd harvest (Figure 4A, B). In general, crotalaria was able to maintain high levels of N-NO_3 in the soil solution until the 2nd harvest, but a high fluctuation was observed along the cycle (Figure 4C, D). When Crotalaria was cultivated under SS+10^{-3} in FOR, a reduction in N-NO_3 in the soil solution was observed across both 1st and 2nd cycles, reaching low levels in the end of the 2nd cycle (Figure 4C). On the other hand, when Crotalaria was cultivated under NS in FOR, after the 1st harvest, an increment of N-NO_3 in the soil solution was observed, which was maintained for three weeks, then dropped until the 2nd harvest (Figure 4C). When Crotalaria was cultivated under CUL, the high levels of N-NO_3 in the soil solution under NS dropped until the 1st harvest, and in the begging of the 2nd cycle an increment in N-NO_3 in the soil solution was observed for about four weeks, then dropped to low levels, until the 2nd harvest (Figure 3D). On the other hand, an increment of the low levels of N-NO_3 in the soil solution was observed under SS+10^{-3} during the 1st cycle, and although a drop was observed, the high levels of N-NO_3 in the solution were maintained until about half of the 2nd cycle, when the N-NO_3 reduced to low values and kept low until the 2nd harvest (Figure 4D).

Regarding the ^{31}P, for both brachiaria and crotalaria, the variation observed were mainly affected by the ^{31}P application, increasing the ^{31}P in the soil solution (Figure 5). Crotalaria under FOR, SS+10^{-3} and ^{31}P application was able to keep higher levels of ^{31}P in the soil solution during the 1st cycle, but in the 2nd cycle, the level of P dropped until the 2nd harvest (Figure 5C). When crotalaria was cultivated under CUL, under P application, both NS and SS+10^{-3} showed higher levels of ^{31}P in the soil solution until the 2nd harvest (figure 5D). ^{33}P was evaluated as well; however, the activity in the solution was very low, close to the equipment detection limit (data no shown).

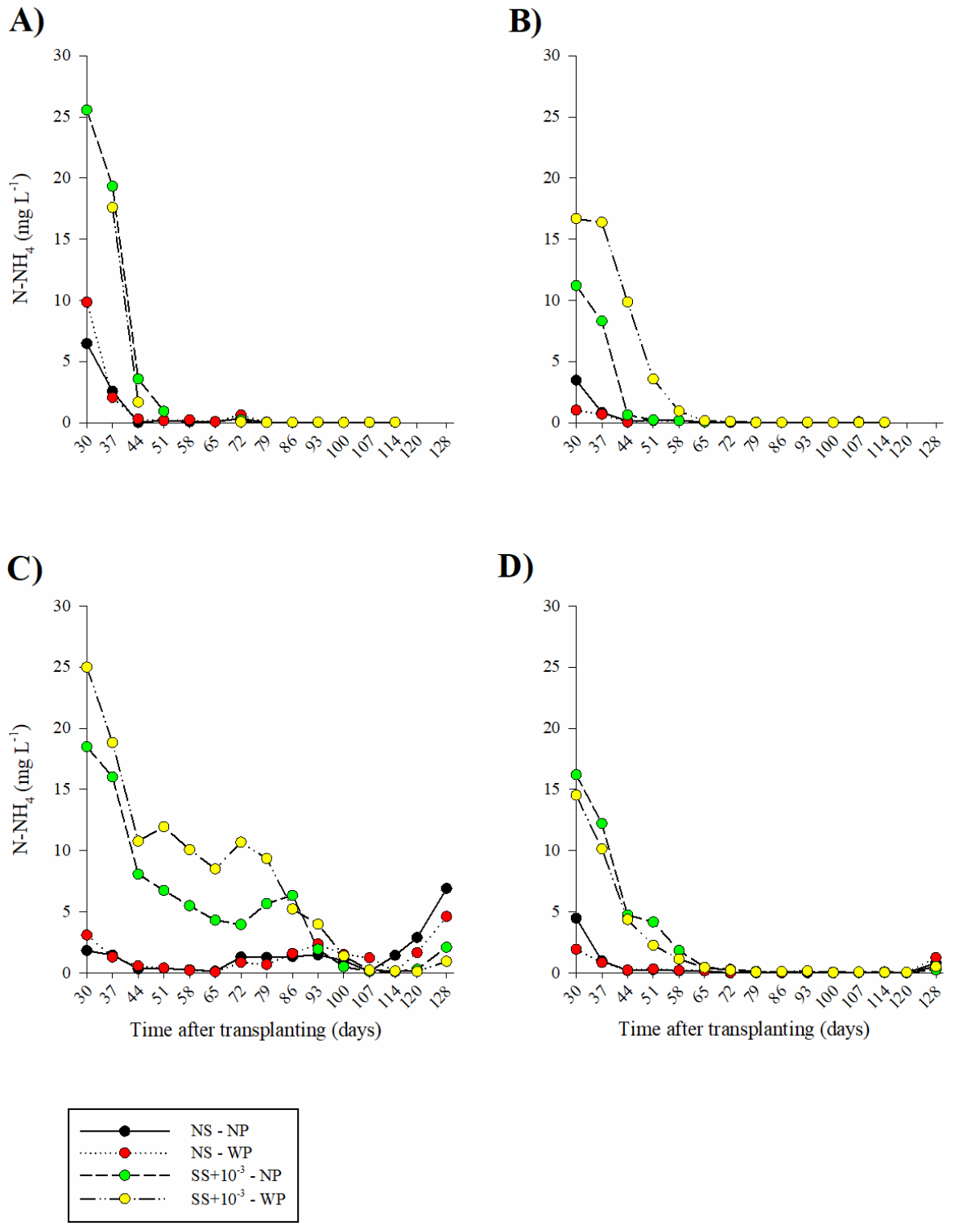

Figure 3. N-NH_4 in the soil solution collected weekly between 30 to 128 days after transplanting of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (*Crotalaria juncea* L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} ($SS+10^{-3}$); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

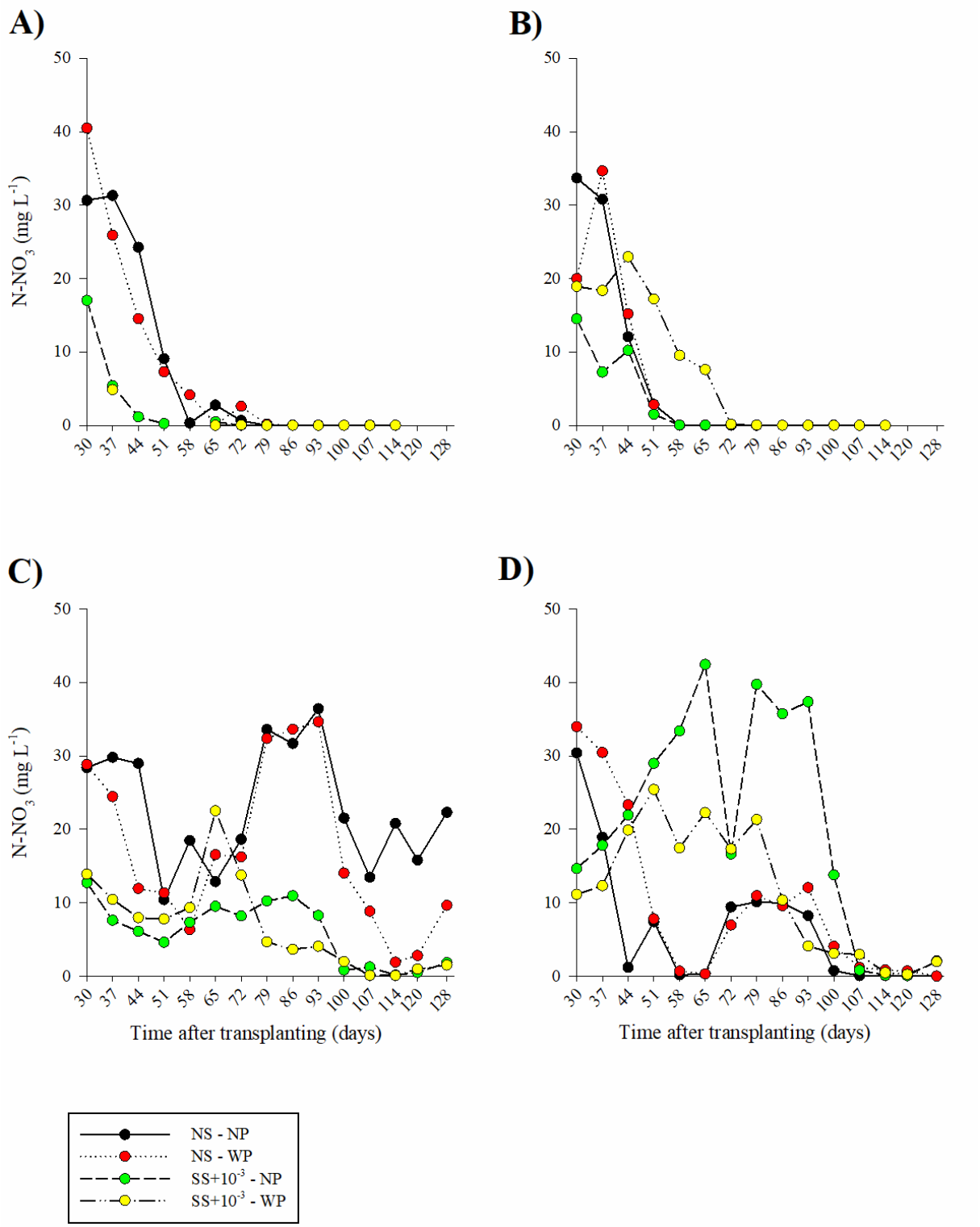

Figure 4. N-NO_3 in the soil solution collected weekly between 30 to 128 days after transplanting of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (*Crotalaria juncea* L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} ($SS+10^{-3}$); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

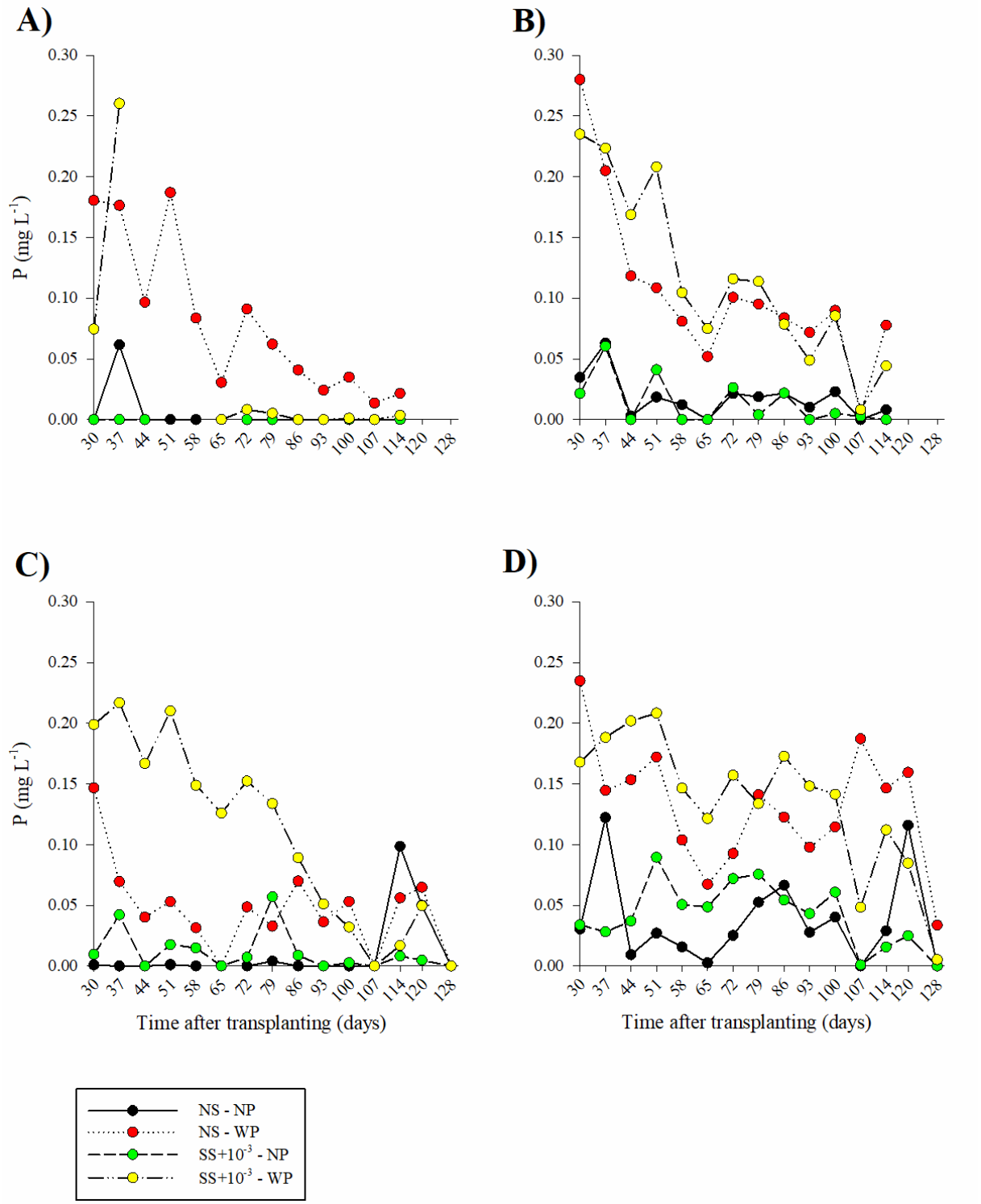

Figure 5. P in the soil solution collected weekly between 30 to 128 days after transplanting of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (*Crotalaria juncea* L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (P)

6.3.3 Plant response to the soil manipulation and P application

Brachiaria, in the 1st harvest, when cultivated under either FOR or CUL, showed higher shoot dry matter under SS+10^{-3} compared to the NS, and the lowest dry matter were observed when brachiaria was cultivated under no P application in NS (Figure 6A, B). For the 2nd harvest, when brachiaria was grown in FOR, no difference was observed for the shoot dry matter between NS and SS+10^{-3}, and P application. For the 2nd cut, in the CUL, the highest shoot dry matter was observed under SS+10^{-3} with P application, and the lowest shoot dry matter were observed under NS, regardless the P application.

For crotalaria, in the 1st harvest, no difference was observed for ^{31}P uptake and shoot dry matter among the soil manipulation treatments and P application, for both FOR or CUL (Figure 6 and 7C, D). For the 2nd cut, when crotalaria was grown under FOR, the highest shoot dry matter and ^{31}P uptake were observed under the SS+10^{-3} with P application, and the lowest shoot dry matter was observed under NS and no P application, but no differences were observed between the treatments under CUL.

Regarding to ^{33}P uptake, in general, both brachiaria and crotalaria cultivated under SS+10^{-3} showed higher ^{33}P uptake. The magnitude in brachiaria was greater compared to crotalaria and the values of ^{33}P uptake increased in the 2nd harvest in FOR under SS+10^{-3}, but in lower magnitude for crotalaria (Figure 8A, B). When crotalaria was cultivated under CUL, did not show any difference in ^{33}P uptake for the soil manipulation, neither in the 1st and 2nd harvest (Figures 8D).

For N uptake, brachiaria, cultivated under FOR, regardless the P application, showed higher N uptake under SS+10^{-3} for the 1st harvest, however for the 2nd harvest an opposite trend was observed and higher uptake was observed under NS (Figure 9A). When brachiaria was cultivated under CUL, for both 1st and 2nd harvest the highest N uptake was observed under SS+10^{-3} (Figure 9B). Crotalaria did not show difference for N uptake according to the treatments for both 1st and 2nd harvest (Figure 9C, D).

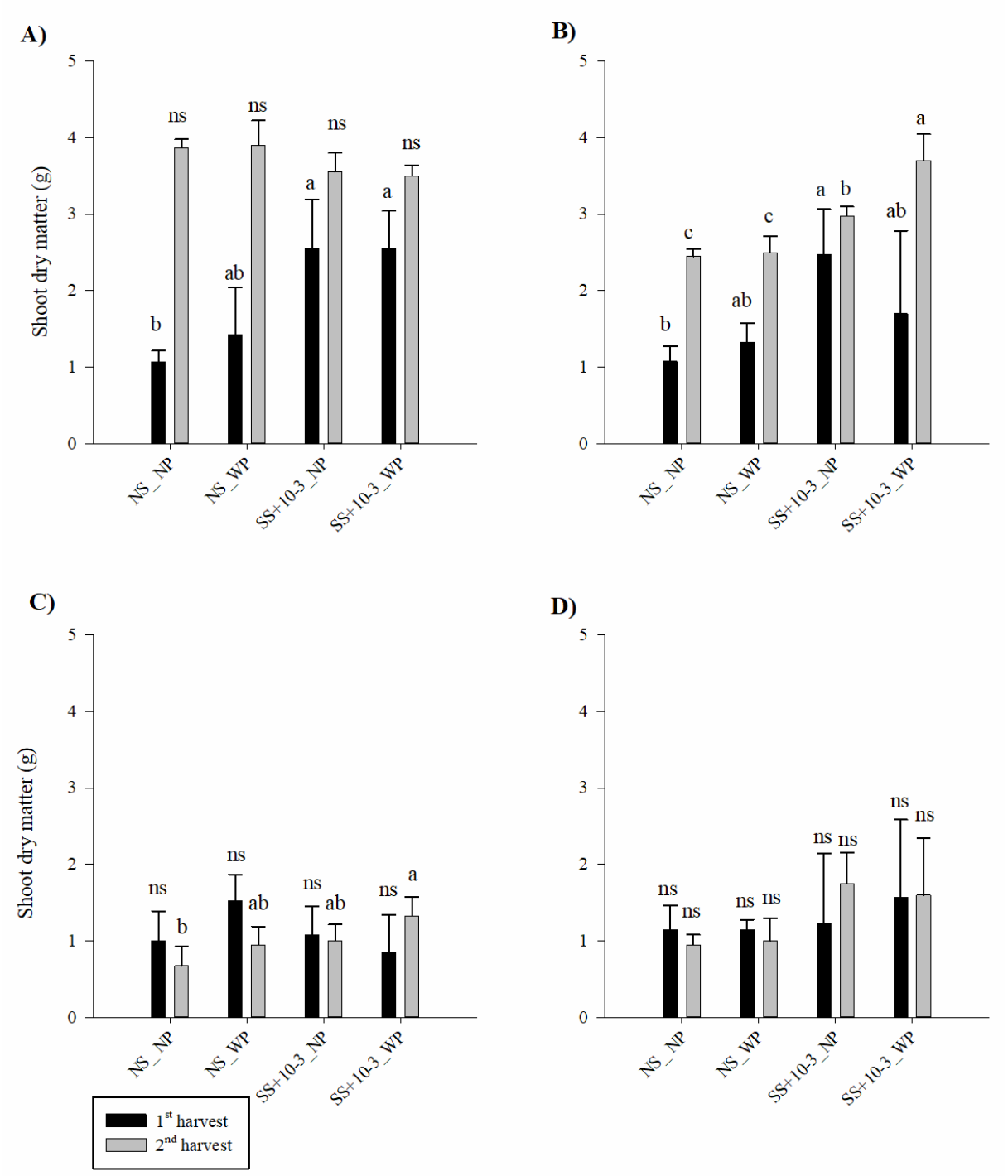

Figure 6. Shoot dry matter of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (*Crotalaria juncea* L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

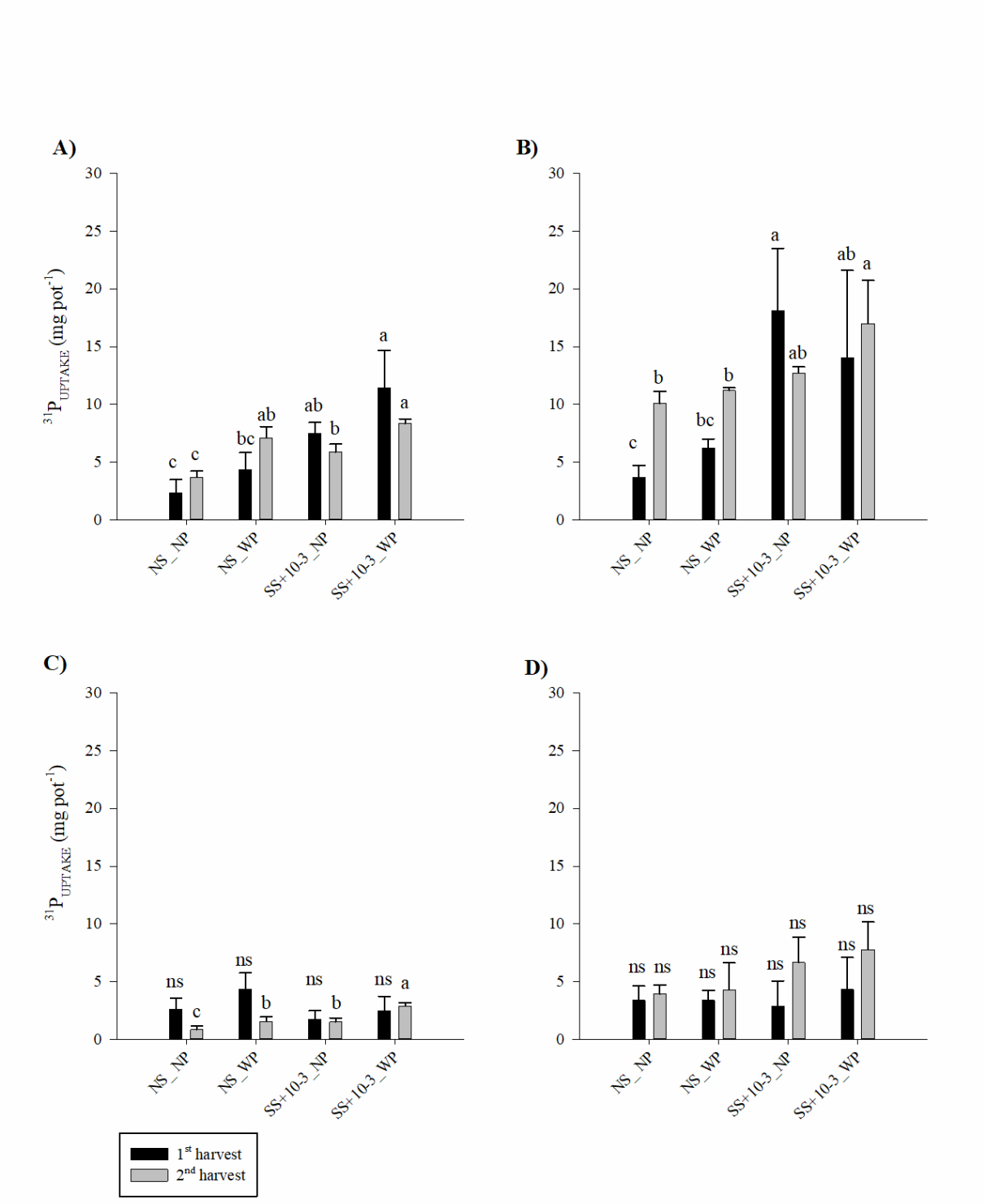

Figure 7. Shoot ^{31}P uptake of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (Crotalaria juncea L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

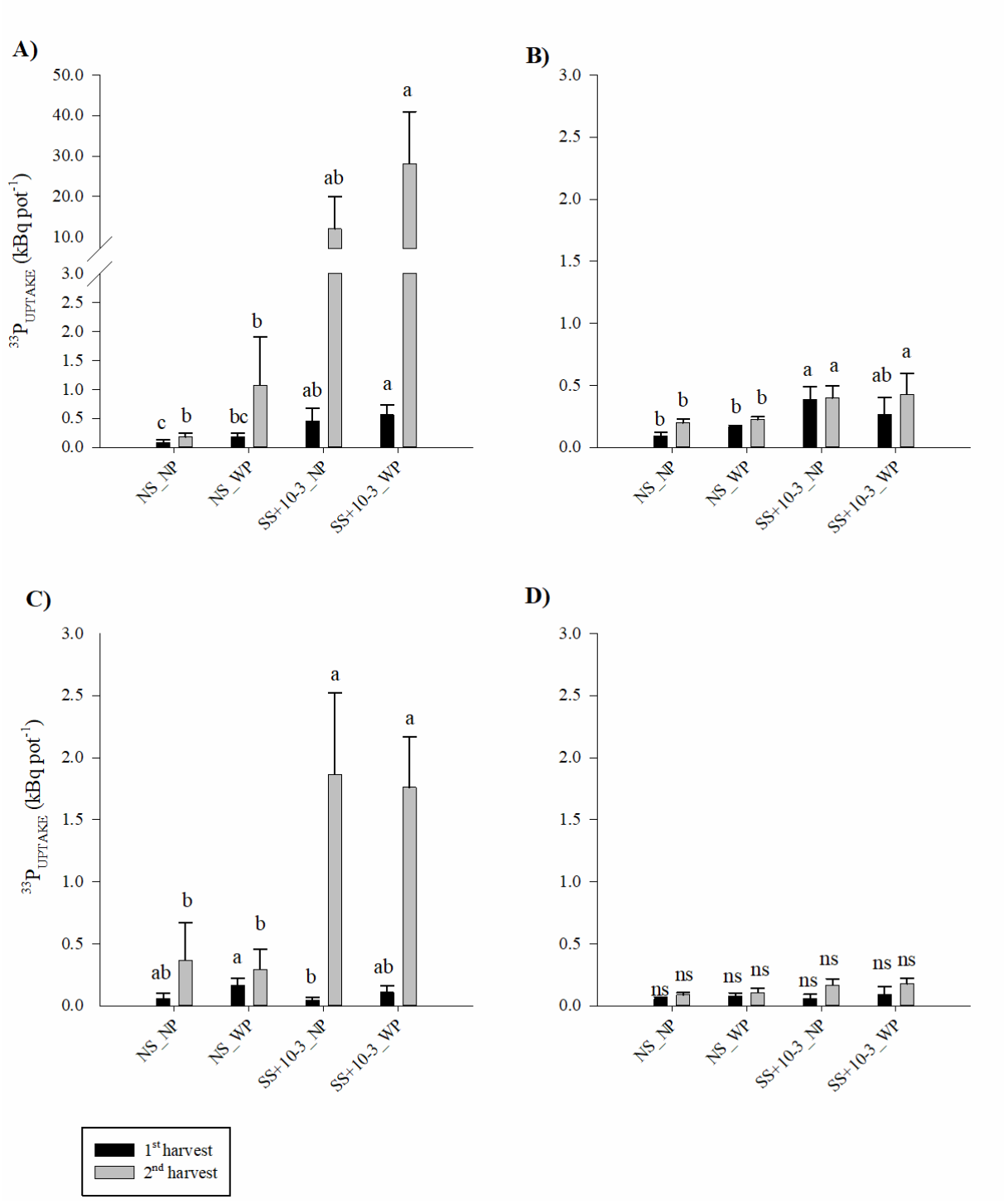

Figure 8. Shoot ^{33}P uptake of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (Crotalaria juncea L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

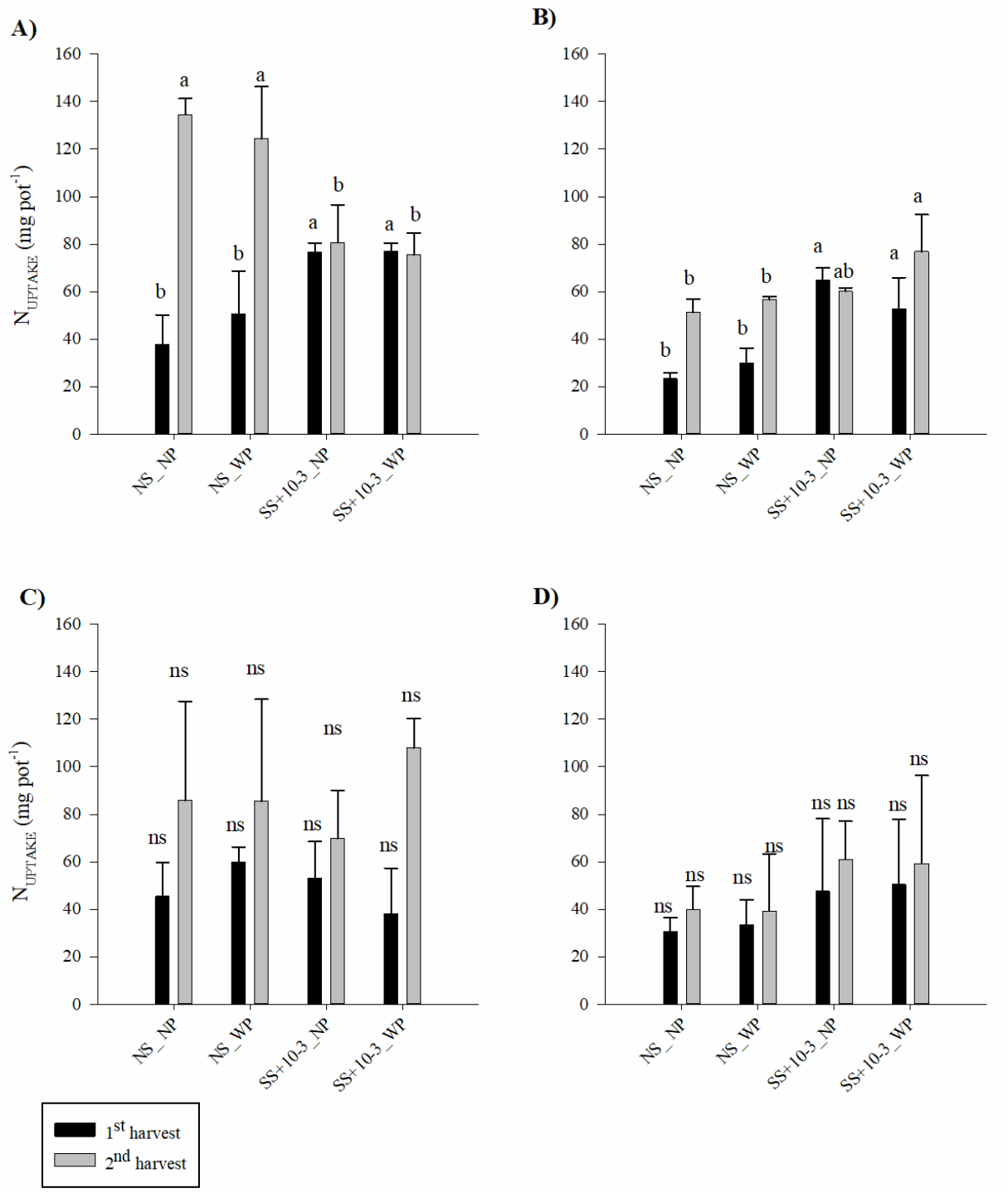

Figure 9. Shoot N uptake of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (Crotalaria juncea L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

6.3.4 P and N in the soil after two crop cycles under microbial manipulation and P application

P concentration in the soil was mainly affected by the P application for both soil (FOR and CUL) and crops (brachiaria and crotalaria) treatments, but not by the microbial manipulation. In magnitude, P was higher in the cultivated soil compared to the forest soil (Figure 10). Regarding to total N in the soil an opposite trend was observed compared to P, and in magnitude, forest showed higher concentration in comparison to cultivated soil (Figure 11).

Brachiaria, under NS and P application in FOR, showed the highest total N in the soil, due to increments of both $N\text{-}NO_3$ and $N\text{-}NH_4$ (Figure 11A). On the other hand, brachiaria under CUL showed the highest total N in the soil under $SS+10^{-3}$ and no P application, with high $N\text{-}NH_4$ (Figure 11B).

Crotalaria did not show any effect of the treatments for total N in the soil, however the proportion between $N\text{-}NO_3$ and $N\text{-}NH_4$ was affected by the treatments. Under FOR, the highest $N\text{-}NO_3$ was observed in NS, regardless the P application. On the other hand, high $N\text{-}NH_4$ was observed under $SS+10^{-3}$ (Figure 11C). Under CUL, crotalaria showed higher $N\text{-}NH_4$ in comparison to $N\text{-}NO_3$, that showed very low values. Then, the contribution of total N in the CUL after two cycles with crotalaria was due to high $N\text{-}NH_4$ content (Figure 11D).

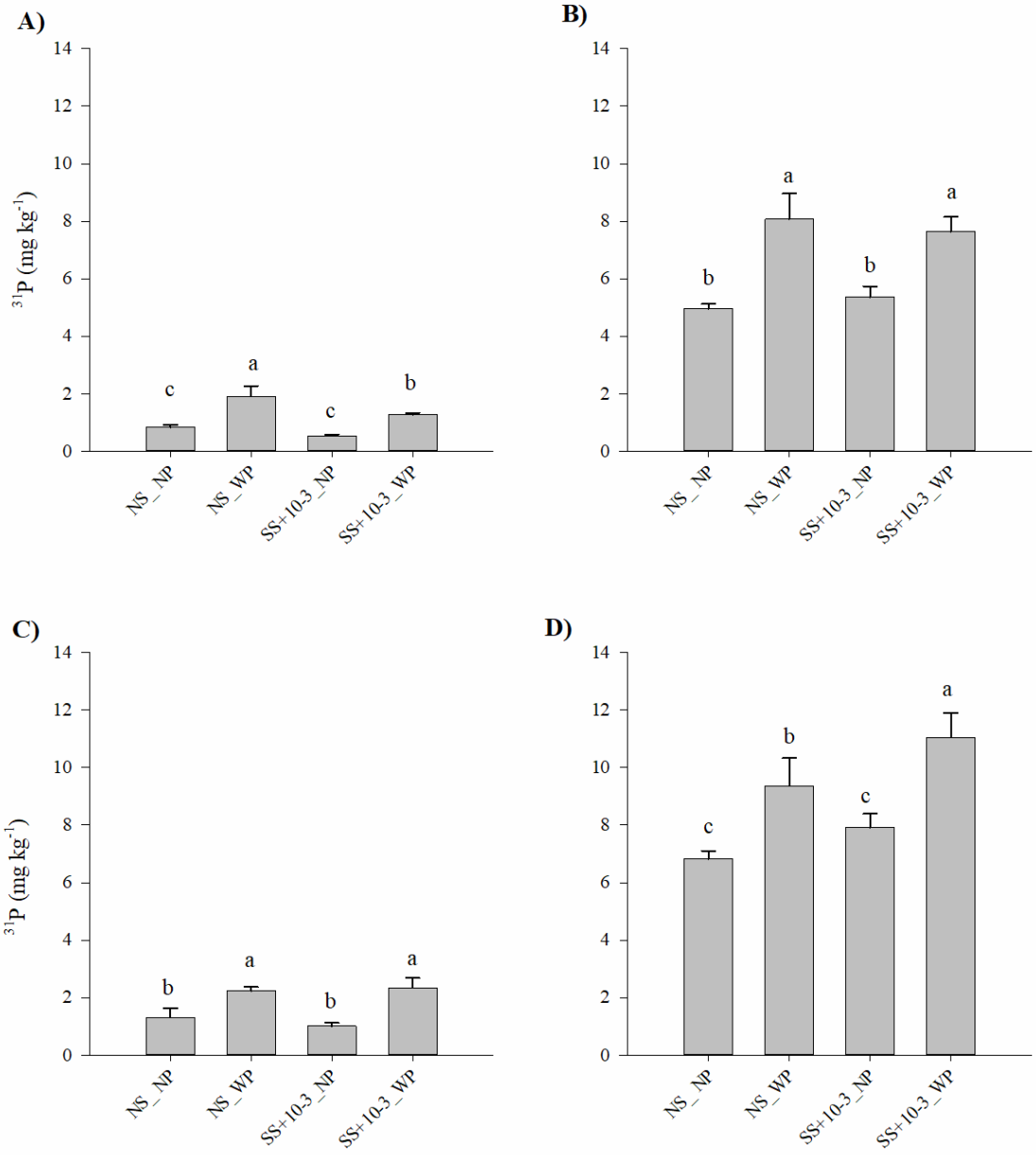

Figure 10. ^{31}P in the soil of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (Crotalaria juncea L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

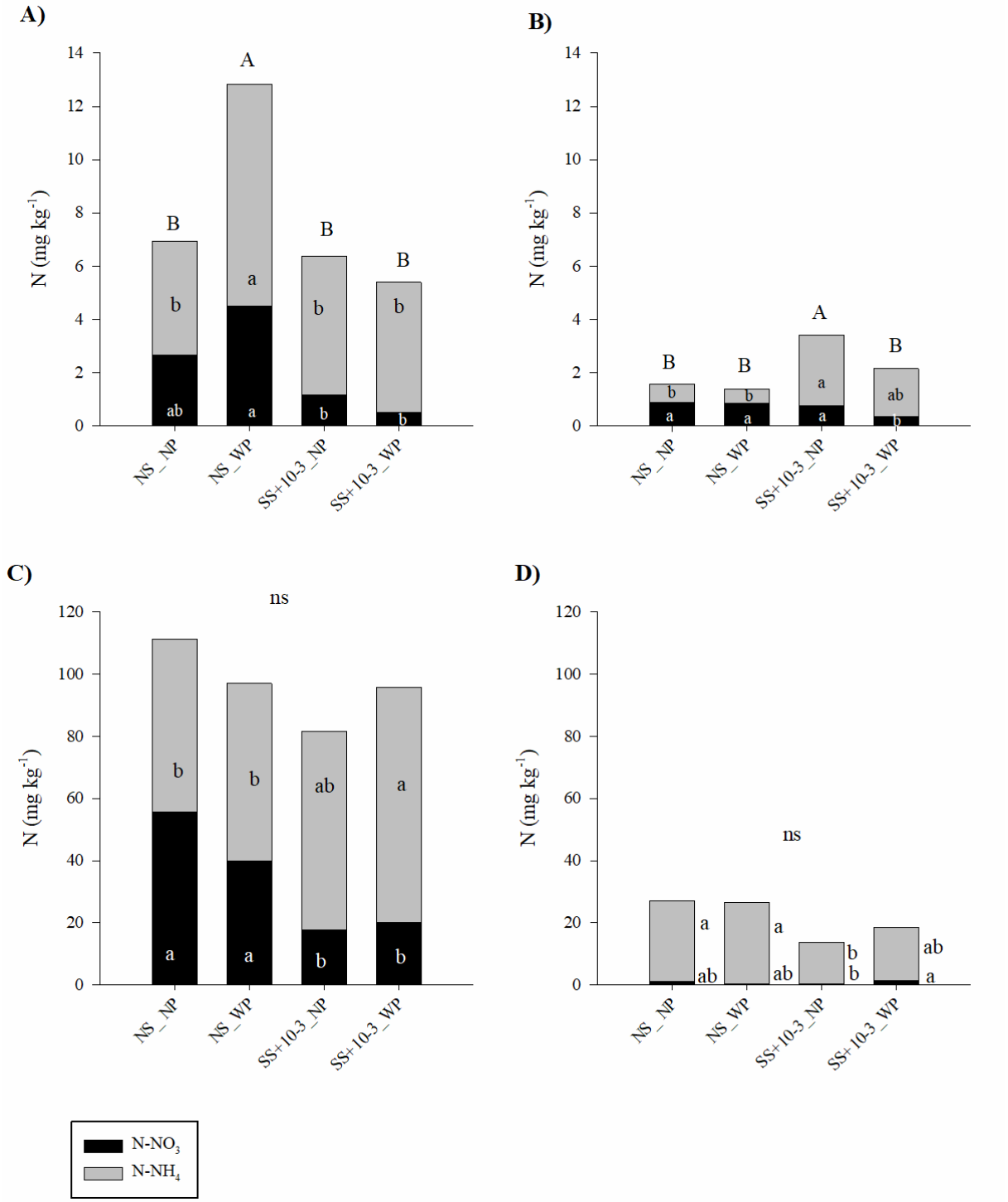

Figure 11. N ($N\text{-}NO_3$ + $N\text{-}NH_4$) in the soil of: **A)** and **B)** brachiaria (*Urochloa brizantha* cv. Marandu) or **C)** and **D)** crotalaria (Crotalaria juncea L.) in a glasshouse experiment using soil from **A)** and **C)** forest (FOR) (~20 years old) and **B)** and **D)** cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} ($SS+10^{-3}$); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

6.3.5 Manipulation of the soil and cropping effect: bacterial composition shifts

After P application and two cropping cycles, the sequencing data showed a reduction in the abundance of β-proteobacteria (Oxalobacteracea), compared to the initial soil (Figure 12). Compared to crotalaria, brachiaria, in general showed lower abundance of all the microrganisms. Crolataria under FOR and NS increased the abundance of Alicyclobacillus and under CUL and $SS+10^{-3}$ increased the abundance of Lysobacter (Figure 12).

In general, for both brachiaria and crotalaria, CUL and FOR, the NS showed higher richness and diversity compared to $SS+10^{-3}$, and no difference for P application, except for crotalaria cultivated in FOR, that did not show difference for richness and diversity between NS and $SS+10^{-3}$ (Table 2)

Bacterial community associated with brachiaria cultivation under FOR was clearly marked by the high ^{33}P uptake, but higher ^{31}P uptake was observed for brachiaria under CUL. According to soil features, the bacterial community of crotalaria, under FOR was defined by the higher $N\text{-}NO_3$ as $N\text{-}NH_4$ in the soil, and thus, high N uptake, and under crotalaria under CUL showed affinity with high P in the soil (Figure 13).

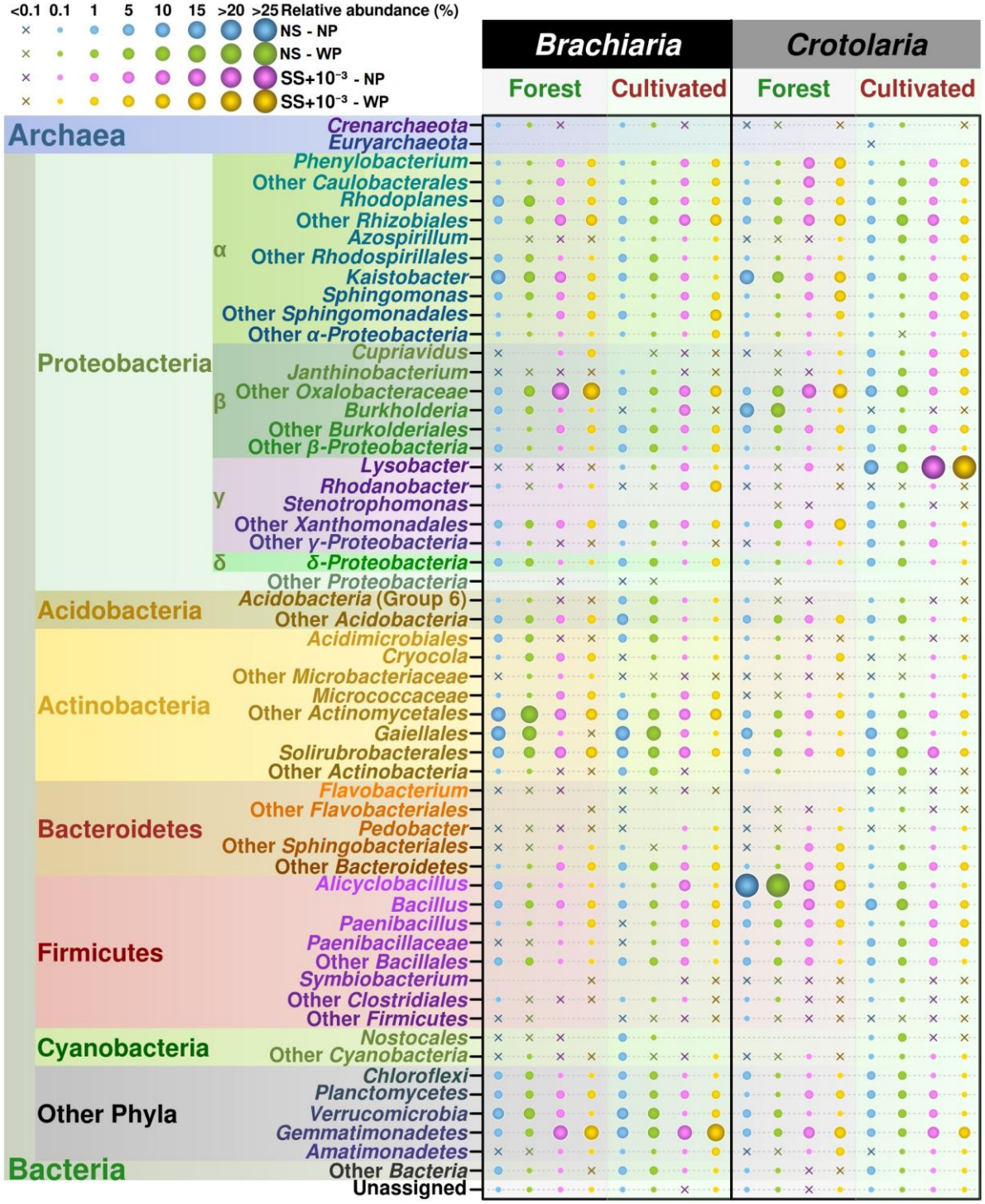

Figure 12. Relative abundande for bacteria community of soil sample cultivated with brachiaria (*Urochloa brizantha* cv. Marandu) or crotalaria (Crotalaria juncea L.) in a glasshouse experiment using soil from forest (FOR) (~20 years old) and cultivated (CUL) area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

Table 2. OTU richness and Shannon (H') index for bacteria of soil sample cultivates with brachiaria (*Urochloa brizantha* cv. Marandu) or crotalaria (Crotalaria juncea L.) in a glasshouse experiment using soil from forest (FOR) (~20 years old) and cultivated (CUL) area (maize), submitted to microbial manipulation.

Treatment	OTU richness		Shannon (H')	
	Brachiaria	Crotalaria	Brachiaria	Crotalaria
	----------Forest----------			
NS - NP	1595 a	825 ^{ns}	5.77 a	4.14 ^{ns}
NS - WP	1562 a	1074	5.75 a	4.29
SS+10^{-3} – NP	946 b	748	4.64 b	4.60
SS+10^{-3}– WP	859 b	788	4.61 b	4.67
	----------Cultivated----------			
NS – NP	1963 a	1485 a	6.61 a	5.71 a
NS – WP	1824 a	1224 a	6.54 a	5.77 a
SS+10^{-3} – NP	1267 b	731 b	5.48 b	4.45 b
SS+10^{-3} - WP	1188 b	641 b	5.40 b	4.28 b

Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

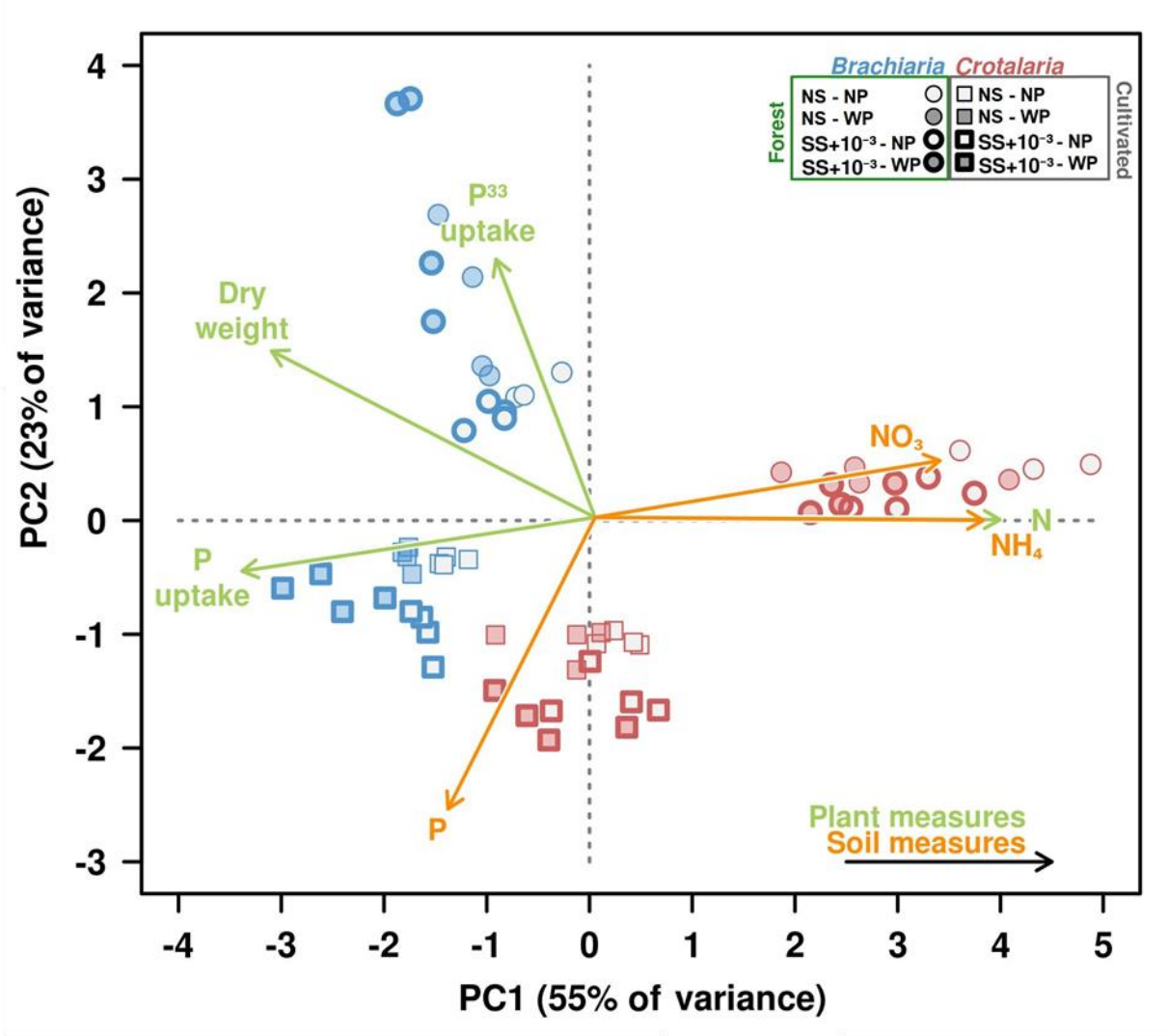

Figure 13. Principal component analysis (PCA) biplot for bacteria of soil sample cultivates with brachiaria (*Urochloa brizantha* cv. Marandu) or crotalaria (Crotalaria juncea L.) in a glasshouse experiment using soil from forest (~20 years old) and cultivated area (maize), submitted to microbial manipulation. Treatment abbreviations are defined as follows: natural soil (NS); sterilized soil by autoclaving twice (121 ºC, 103 kPa, 1 h) (SS), followed by inoculation of dilutions 10^{-3} (SS+10^{-3}); no P application (^{33}P-water) (NP) and P application (^{33}P-at rate of 60 mg kg^{-1}) (WP)

6.4 Discussion

6.4.1 Alteration on bacteria community and chemical features in soil from forest and cultivated area with microbial manipulation

The integration of bacterial sequencing data and environmental variables showed that the separation of the bacteria between FOR and CUL was marked by the high chemical parameters in the soil observed in the CUL, compared to the FOR, indicating that those soils

present naturally different scenarios, in regarding to chemical features, that modulate the microbial community itself.

Forest has no input of fertilizer along the time, so the nutrient cycling is responsible to provide the essential nutrients to plants in this environment, while the cultivated soil receives fertilizer every year to supply the nutrient needs of the crops. This modulate the microbial community differently. Furthermore, changes in the soil microbiome may occurs when plants are cultivated in the soil, because they interact with microorganisms around, and the simple fact of the soil use for agriculture can promote a microbial selection in an environment, changing the soil biodiversity and agroecosystem functions (Giller et al. 1997).

Situations of extreme disturbs, such as wildfire, also may cause losses in level of soil microbial diversity for both, bacteria (Ferrenberg et al. 2013; Smith et al. 2008) and fungi (Whitman et al. 2019). These changes may reflect in chemical changes as well.

Our results showed that the manipulation of both soils using autoclaving and inoculation of 10^{-3} (SS+10^{-3}) affected most of the chemical features, including an increament in N-NH_4 compared to the NS, that present higher N-NO_3, as observed in the previous chapter of this thesis.

6.4.2 Response of brachiaria cultivated in manipulated microbiome and P application

Brachiaria showed different trends between cultivation in FOR and CUL, according to the microbiome manipulation.

When brachiaria was cultivated in the FOR, in the 1st harvest, the highest dry matter, P and N uptake were observed under SS+10^{-3}, regardless the P application, showing similar results obtained in the previous chapters of this thesis. In this situation N was detected in the soil solution, both N-NH_4 and N-NO_3. Therefore, nutrients were available to the plants, and under no competition with the microorganisms, promoted by the soil autoclaving and inoculation of 10^{-3} community, brachiaria was able to develop well.

After the 1st harvest, the levels of N in the soil solution dropped and were not in the soil solution during the 2nd cycle, in both NS and SS+10^{-3}. Analysing the nutrients remained in the soil after the 2nd cycle of brachiaria, NS showed higher total N remain in the soil compared to the SS+10^{-3}, both NO_3 and NH_4. Consequently, in the 2nd harvest, brachiaria cultivated under NS showed higher N uptake, regardless the P application, compared to the SS+10^{-3}.

Brachiaria in the 1st cycle depend on the nutrient sources from the seed, soil and the formation of shoot to promote the photosynthesis. However, due to the capacity of brachiaria

to regrow, even the photosynthesis is the most important source of assimilates for the plant, after a severe shoot cut and no leaves left, the roots and the steam seem to act as source of non-structural carbohydrates as substrate for the respiration, until the shoot is re-established (May 1960; Pimentel et al. 2016). In conditions where the N is limiting, the CO_2 and N reserves remobilization is higher (Alexandrino et al. 2008; Skinner et al. 1999). That is why even with low levels of N detected in the soil solution for the 2nd cycle, brachiaria was able to increase the rate of growth. However, the P uptake was higher under P application, for both NS and SS+10^{-3}. Therefore, in the 2nd harvest, the balance between the high N uptake under NS and high P uptake under NS resulted in no difference for brachiaria shoot dry matter between NS and SS+10^{-3}. Thus, even with more N uptake, the NS was not able to overcome the dry matter production compared to the SS+10^{-3} in the 2nd cycle.

This result of brachiaria cultivated under FOR and NS after the 2nd cycle may be an overcoming of the competition between plant and microorganisms for N. Kuzyakov and Xu (2013) showed the ecological importance of the competition and highlighted the fact that even microorganisms and plants compete for the same resource, such as N, the microbial biomass may prevent the N losses by leaching during periods of slow or no root uptake; and may be a source of N for the roots according to plant demand; and evolutionary development culminating in a mutualistic interaction between roots and microorganisms. This was evident in FOR and NS, where, during the 1st cycle the microorganisms took advantage in relation to the plant, but in the 2nd cycle, due to the regrowth of brachiaria and that the rhizosphere microbial community was adapted for this crop, in the 2nd cycle this effect was softened.

On the other hand, when brachiaria was cultivated in CUL, for both 1st and 2nd harvest, the highest values of shoot dry matter, P uptake and N uptake were observed under SS+10^{-3}, regardless the P application. Li et al. (2019) indicates that even after sterilization of the soil, the healthier organisms are rearranged by the plants.

Under CUL and SS+10^{-3} brachiaria showed high total N remained in the soil. This condition showed low richness and diversity, but the plants showed the highest shoot dry matter. This reinforces the results observed in the previous chapters of this thesis, where brachiaria present high P uptake and shoot dry matter production under SS+10^{-3}. Brachiaria is adapted to regrow and is tolerant to fire. In case of fire, the microorganisms around may be affected, but the flammability may fit the microbial community, resulting in the death of the neighbouring less flammable individuals and also open recruitment possibilities (Bond and Midgley 1995).

In an overview, if we compare the OTU number after two cycles of brachiaria, the highest was observed under CUL and NS, were plants did not grow well. On the other hand, the reduction of OTU under CUL and SS+10^{-3} favoured the brachiaria growth. Considering the FOR, in NS, the OTU number was comparable to the CUL under SS+10^{-3}, and so favoured the plant due to the reduction of the competition in N uptake, and the plant developed better, while FOR under SS+10^{-3}, low N uptake was observed, but no reduction in shoot dry matter.

6.4.3 Response of crotalaria cultivated in manipulated microbiome and P application

Crotalaria, in general, did not show differences in shoot dry matter, P uptake and N uptake, cultivated either under FOR or CUL. Therefore, SS+10^{-3}, for both FOR and CUL, did not affect the development of the plant and in relation to the soil solution, and even increased the levels of N-NO_3 in the soil solution under application of P in SS+10^{-3} compared to NS.

Considering the effects on the microbial community, under FOR, crotalaria did not show difference in richness and diversity between NS and SS+10^{-3} and under CUL a reduction in these indexes as observed under SS+10^{-3} compared to NS, however, these results did not affect the shoot dry matter of this crop. The lack of response on soil modifications on crotalaria response was also observed in the previous chapters of this thesis.

Eo et al. (2015) showed that despite the benefits brought by the *Crotalaria juncea* as a cover crop, such as high biomass and N production, the bacteria PFLA concentration was lower compared to *Sorghum bicolor* var. sudanense.

Regarding the effect of crotalaria on the nutrient remained in the soil, generally, crotalaria was able to keep N and ^{31}P in the soil solution for both cycles. ^{31}P was higher under P application, but the total N did not show difference according to the treatments, and the FOR the total N was higher compared to CUL. Under FOR, crotalaria maintained the levels of N similar to the initial, after microbiome manipulation, but the proportion between the N-NO_3 and N-NH_4 was more equitable amounts, indicating that assimilatory reduction is happening in the NS and nitrification in the SS+10^{-3}. While under CUL in NS, the total N clear reduced, and the conversion of N-NO_3 to N-NH_4. On the other hand, SS+10^{-3} in the begging was predominant the NH_4 and after the cropping a reduction was observed in the total N, but NH_4 still counted for most of it. This indicates that no nitrification is occurring and most of NH_4 was consumed under CUL and SS+10^{-3}. However, regardless the N transformations in the soil, the crotalaria shoot production was not affected.

6.5 Conclusion summary

Our results showed that the N was relevant to determine the plant development, compared to P. These results are supported by Oliveira et al. (2001) who observed that in degraded area, N was more important than P. In our case, this indicated that the groups microorganisms that were present in the soil after microbial manipulation determined the fate of the N, and so the development of the plant, but the P application was not that much determining.

Furthermore, the effect of crop was evident. Both brachiaria and crotalaria, after cropping, created a propitious scenario regarding to the microorganisms according to their needs in the soil. While brachiaria, a Poacea, has the regrowth characteristic after a cut, what may increase the plant competition by resources, crotalaria, hence, is a legume and so can fix N from the atmosphere. Both crops are widely used as green manure and cover crops. Crotalaria showed that the reduction on microbial diversity did not decrease its developments and brachiaria cultivated in as in microbial degraded area showed superiority in growth under $SS+10^{-3}$, which represent alternatives for improve the soil qualities for the next crop in an agriculture system, even in degraded area. Peter et al. (2011) says that more important than the diversity is the community composition.

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7 FINAL CONSIDERATIONS

With this thesis we evaluted the effect of soil microbial manipulation on the interaction between soil and plant.

In this sense, experiments at grasshouse and laboratory are more proned to precisely study the role of microbial diversity when compared to field conditions, once the controlled parameters of the samples are more easily monitored.

In the chapter 2 of this thesis, we generated different soil microbial communities and our results showed that different microbial diversity extinction methods generated different microbial communities evaluated by the sequencing, but the microbial diversity of the non manipulated soil was similar to the sterile soil. On the other hand, the acid phosphatase activity reduced in a gradient according to the severity of the stress that the soil was submited, and non manipulated soil showed opposite trend compared to the sterile soil. When crops were cultivated in those different conditions, brachiaria showed higher dependency on microbial community, but crotalaria was not harmed by the reduction on microbial diversity for micorrhization. In parallel, due to the gradient of mycorrhization in the roots obtained in the experiment described in the chapter 2, we were able use the samples to test the correlation between different methodologies to determine the rates of colonization, which was present in the chapter 3. In this chapter we concluded that is possible to use qPCR to predict the mycorrhization on root samples.

After evaluation of the effect of the soil microbial changes on the mycorrhization, we evaluated the manipulation of soil microbial community on the plant P uptake. For this, in the chapter 4 we evaluated the effect of levels of diversity for the microbial community on soils, upon microorganisms and plant dry matter production. As observed in the chapter 2, the acid phosphatase activity was reduced in the manipulated conditions. Regarding the plant response, we observed that after sterilization of the soil using autoclaving, the soil are able to recover and provide condition to growth plant, likely due to the microbial community according to the plant need. This shows the resilient properties that soil shows when submitted to a stress condition and the capacity to recover.

Due to the similarity on non manipulated soil and sterile soil regarding to microbial diversity obtained by the sequencing, observed in the chapter 2, and the possible interferences of the autoclaving procedure in the soil chemical attributes, in the chapter 5 is described an experiment that evaluated the autoclaving procedure, to undestand the changes in microbial and chemical features. We observed that in autoclaved soil $^{14}CO_2$ emission via microbial respiration was null, and no change in the total N was observed, but there was an increase in

$N\text{-}NH_4$ compared to $N\text{-}NO_3$. In addition, the autoclaving procedure does not alter soil ^{31}P concentration, but reduces labile ^{33}P activity. Therefore, although the autoclaving is an easy and practical procedure to promote sterilization of the soil, it may promote changes in the chemical attributes, and so affect the nutrient dynamics. Alternative methods to sterilize the soil must be evaluated to promote the elimination of the microorganisms, but not affecting the chemical attributes.

Due to the effect of autoclaving in the soil N transformations observed in the chapter 5 of this thesis, we evaluated the effect of changes promotes in the microbial community using autoclaving in the P and N cycling of different crops. As results, in the chapter 6 we observed that bachiaria increased dry matter production, N and P absorption when cultivated under soil that suffered microbiome reduction during the 1st cycle, as observed in the chapter 4. For the 2nd cycle, brachiaria increased N uptake but did not reflect increase in shoot dry mass when cultivated in manipulated soil compared to soil without microbial manipulation. On the other hand, crotalaria was not affected by microbiome manipulation for shoot dry matter, N and P absorption, as observed in the chapters 2 and 4.