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Molecular bioindicators prospection from maize rhizosphere microbiota to N_2O gas mitigation under stover coverage

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to my wife
Karine

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ABSTRACT

YOSHIURA, C. A. **Molecular bioindicators prospection from maize rhizosphere microbiota to N_2O gas mitigation under stover coverage**. 2018. 86 f. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2018.

The corn crop is one of the most important crop in the world and several studies for production and yield increment have been done, but there is a lack of information about inputs combinations and interactions among microorganisms and plant roots over greenhouse gases (GHGs) emissions. Therefore, the understanding of the effects and prospect these microorganisms' activities can provide applicable knowledge in return, producing bioproducts to improve agriculture managements. All information about microbes and their way of life are essential to looking for certain groups of microorganisms through molecular techniques like gas chromatography, quantitative PCR (qPCR), next-generation sequencing (NGS), and isolate them by classical microbiology techniques. Thus, this thesis was defined to cover a complete work from evaluate inputs used in corn crop and prospecting microbial bioindicators until isolation methods for N_2O mitigation that could be applied in new commercial bioproducts in the future. Therefore, the objective purposed was to evaluate urea topdressing fertilization, microbial inoculant and maize stover interactions with rhizosphere communities in Brazilian tropical soil under controlled conditions experiments, in order to identify bioindicators for nitrous oxide (N_2O) mitigation. In continuity, a lignocellulose-rich soil (Amazonian Dark Earth – ADE) was selected for comparative analysis with corn field soils (CFS) to understand maize stover degradation process and its GHGs fluxes relations; and, finally, to perform microbial isolation methods in modified media under hypoxic condition. The results revealed two or more inputs in combination can merge individual characteristics of each or potentialize their effects. The two-variable microbial inoculant and maize stover coverage combination (IS) showed as the best cost-benefit for plant biomass production (at least 10.8% more than other two- and single-variable treatments) and GHGs mitigation (12.1% less emissions than all-amendments combination treatment – i.e. FIS – which retain similar biomass production) due to interactions at rhizosphere level that revealed increment in *Actinobacteria* (21% to 37%) and *Firmicutes* (2% to 10%) phyla representativeness in comparison to other treatments. The effects derived from cellulose degradation were observed from metatranscriptome analysis that revealed an avoidance of N_2O production (up to 3-fold of expression) by other pathways activities from both soils (ADE and CFS) with more intense gene expression response in ADE soils. In consequence, culture media modified for isolation of denitrifiers successfully obtained most of strains belonging to *Actinobacteria* and *Firmicutes* phyla as *Bacillus*, *Paenibacillus*, *Arthrobacter* and *Streptomyces*. In conclusion, *Actinobacteria* and *Firmicutes* showed as bioindicators for GHGs mitigation under maize stover coverage.

Keywords: Microbial molecular ecology. Bioinformatics. Nitrogen cycle. 16S. Shotgun sequencing

RESUMO

YOSHIURA, C. A. **Prospecção de bioindicadores moleculares da microbiota na rizosfera do milho sob efeito de palhada na mitigação do gás N_2O.** 2018. 86 f. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2018.

O cultivo de milho é uma das mais importantes culturas do mundo e vários estudos visando o aumento da produção e da produtividade têm sido realizados, todavia existe uma carência de informação sobre as combinações de aditivos e das interações entre os microrganismos e as raízes das plantas frente a emissão de gases do efeito estufa (GEEs). Dessa forma, o entendimento dos efeitos e a prospecção das atividades desses microrganismos podem prover o retorno de conhecimento aplicável, produzindo bioprodutos em favor da melhora dos manejos agrícolas. Toda informação sobre os microrganismos e seus modos de vida são essenciais na busca de certos grupos através de técnicas moleculares, como cromatografia gasosa, PCR quantitativo (qPCR) e o sequenciamento de nova geração (NGS), e então realizar o isolamento destes pelas técnicas de microbiologia clássica. Assim, essa tese foi delineada em abranger um trabalho completo desde a avaliação dos aditivos usados no cultivo de milho e a prospecção de bioindicadores microbianos até os métodos de isolamento para a mitigação de N_2O que podem futuramente serem aplicados em novos bioprodutos comerciais. Portanto, o objetivo proposto foi avaliar as interações entre fertilização de cobertura com ureia, o inoculante microbiano e a palhada do milho com as comunidades da rizosfera do milho em solo tropical brasileiro sob experimento em condições controladas, com o intuito de identificar bioindicadores para a mitigação do óxido nitroso (N_2O). Em continuidade, o solo rico em lignocelulose (Terra Preta da Amazônia – TPA) foi selecionado para análise comparativa ao solo de campo de milho (SCM) para entender o processo de degradação da palhada do milho e sua relação aos GEEs; e, por fim, realizar o os procedimentos de isolamento microbiano em meio de cultura modificado sob condições hipóxicas. Os resultados revelaram que a combinação de dois ou mais aditivos podem mesclar as características individuais ou potencializar seus efeitos. A combinação de duas variáveis (inoculante microbiano e a cobertura de palhada do milho – IS), mostrou-se o melhor custo-benefício para o ganho de biomassa da planta (pelo menos 10,8% mais que outros tratamentos com duas ou uma variável) e mitigação de GEEs (12,1% menor que o tratamento completo – FIS – que retém produção de biomassa similar) devido a interações em nível rizosférico que revelaram um incremento em representatividade nos filos *Actinobacteria* (de 21% para 37%) e *Firmicutes* (de 2% para 10%) em comparação aos demais tratamentos. Os efeitos derivados da degradação da celulose foram observados nas análises de metatranscritoma com uma evasão na produção de N_2O (até 3 vezes de diferença) por atividades em outras vias de ambos os solos (ADE e CFS) havendo maior intensidade de resposta na expressão dos genes em ADE. Por consequência, a modificação do meio de cultura para o isolamento de microrganismos do ciclo do nitrogênio obteve sucesso com a maioria dos isolados pertencendo aos filos *Actinobacteria* e Firmicutes como *Bacillus*, *Paenibacillus*, *Arthrobacter* and *Streptomyces*. Em conclusão, *Actinobacteria* e Firmicutes mostraram serem is bioindicadores para a mitigação de GEEs sob a cobertura de palhada do milho.

Palavras-chave: Ecologia microbiana molecular. Bioinformática. Ciclo do Nitrogênio. 16S. Sequenciamento *shotgun*

LIST OF ABBREVIATIONS

AC	Avicel™ or microcrystalline cellulose (celulose microcristalina)
ADE (TPA)	Amazonian Dark Earth (Terra Preta da Amazônia)
C	Control group
Ca	Calcium
CFS (SCM)	Corn field soil (solo de campo de milho)
CH_4	Methane (metano)
CMC	Carboxymethylcellulose (carboximetilcelulose)
CO_2	Carbon dioxide (dióxido de carbono)
CO_2e	Carbon dioxide equivalent (dióxido de carbono equivalente)
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
F	Urea topdressing fertilization at V5 treatment
FI	Combination of F and I treatment
FIS	Combination of F, I and S treatment
FS	Combination of F ans S treatment
GHGs (GEEs)	Greenhouse gas (gases do efeito estufa)
I	*Azospirillum brasiliense* inoculant at seeds sowing treatment
IAA (AIA)	Indole-3-acetic acid (ácido indolacético)
IS	Combination of I and S treatment
K	Potassium
KCl	Potassium chloride
Mg	Magnesium
mRNA	Messenger ribonucleic acid
N_2O	Nitrous oxide (óxido nitroso)

NFCC	Nitrogen-free combined carbon (combinado de carbono livre de nitrogênio)
NGS (SNG)	Next-generation sequencing (sequenciamento de nova geração)
NMDS	Nonmetric multidimensional distance scaling
NPK	Nitrogen, Phosphorus and Potassium formulation
OM (MO)	Organic matter (matéria orgânica)
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction (reação em cadeia da polimerase)
pH	Potential of hydrogen
PGPR (RPCP)	Plant growth-promoting rhizobacteria (rizobactérias promotoras do crescimento de plantas)
qPCR	Quantitative PCR (PCR quantitativa)
rRNA	Ribosomal ribonucleic acid
S	Maize stover coverage treatment
V15	15^{th} mature leaf stage
V5	5^{th} mature leaf stage
WFPS	Water-filled pore space

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1 INTRODUCTION

The corn crop corresponds to the second biggest production of grains in Brazil, after soybeans crop (EMBRAPA, 2015), with highest demand for nitrogen fertilizers (around 1.1 thousand tons) that correspond to 27.4% of total nitrogen fertilizer among Brazilian crops (IFA, 2017). In 2010, the Brazilian agriculture soils released around 452.4 Gg of nitrous oxide (N_2O) to the atmosphere (84.2% from total N_2O emissions), that 35.7 and 39.5 Gg of N_2O (i.e. 10.6 and 11.8 Tg in carbon dioxide equivalent - CO_2e) were emitted from synthetic fertilizers and agricultural waste, respectively; it means, an increment of 29.9% and 35.7% in comparison to 2005 evaluation (BRASIL, 2016). This way, efforts to understand greenhouse gases (GHGs) fluxes from several crop management strategies are being intensively assessed to reduce their emissions directly or indirectly.

Several studies around the world evaluate inputs and their influences in corn crop: microbial inoculant combined to nitrogen fertilizer on GHGs emissions (CALVO et al., 2016; MÜLLER et al., 2016), nitrogen fertilizer on GHGs emissions and crop yields (ABALOS et al., 2016), nitrogen fertilizer on corn yield (CAMBOURIS et al., 2016), nitrogen fertilization and manure on microbial structure (PEIFFER et al., 2013), organic amendments and GHGs (THANGARAJAN et al., 2013). Although, few studies explored effects of nitrogen fertilization, microbial inoculant and plant residue (combined or not) in microbial structure and correlate their functions with GHGs fluxes and crop yield increments.

Many factors are responsible for plant development, specially factors related to the roots that is the main gate of nutrients absorption and substances exudation, for these nutrients exchanges there is a complex interaction among roots and microorganisms. Hence, intense gene expressions occur in maize rhizosphere more than bulk soil (LI et al., 2014), mainly under the “priming effect” that is a short-term change of organic matter (OM) often after inputs of organic or mineral fertilizer or exudation of organic substances by roots that can lead to GHGs emissions, by virtue of the acceleration of microbial metabolism and the respiratory activity (BINGERMAN et al., 1953; THANGARAJAN et al., 2013). The roots of plants can release organic substances during their development and growth, on where several groups are privileged from these and can live closely to this nutrient resource, for example diazotrophic bacteria have been related to colonize roots surface, in return they help plant in development with nitrogen fixation, nitrate reduction and phytohormones production like auxins – e.g. indole-3-acetic acid (IAA) (BOTHE et al., 1992; COSTACURTA et al., 1994; BASHAN et al., 2004). These beneficial interactions of biological processes among root-exudates,

microorganisms and soil amendments can create an environment to enhance plant growth (BADRI and VIVANCO, 2009; THANGARAJAN et al., 2013). For that reason, microbial inoculants have been explored as probiotic in soil-plant interactions to enhance plants growth.

Azospirillum brasiliense is a free-living diazotrophic bacterium with nitrogenase activity for biological N_2 fixation under microaerophilic condition where nitrogen is limited, that has been used in grass crops (like maize, rice and wheat) as commercial microbial inoculant with successful results, increasing plants production and yield (EMBRAPA, 2011). This bacterium, which belongs to Alphaproteobacteria class, has vibroid shape with a single polar flagellum for motility, as known as a plant growth-promoting rhizobacteria (PGPR) through secretion of phytohormones (OKON, 1994; SOMERS et al., 2005; AHEMAD and KIBRET, 2014) and it has other activities and interactions at rhizosphere level including phosphate solubilization, degradation of siderophores, biological control of soil-borne pathogens (BASHAN et al., 2004).

The soil microbiota mediate ecosystem sustainable functioning in nutrients cycling and other biological processes (e.g. mineralization, decomposition and greenhouse gases fluxes), due to this characteristic, microbial groups structure are researches' target as biological indicators for environment health and disease suppression (BRUGGEN and SEMENOV, 2000). However, differences and shifts in physicochemical properties have to be considered to the structure of microbial population resolution among soils (GIRVAN et al., 2003). For microbiota overview, Meta-Omics analyses from any environmental source (such as soil, water, air, and human and animal samples) can provide holistic profile – taxonomically and functionally – to understand biogeochemical cycles and other processes performed by microorganisms, exploring well-known or unknown pathways (PETROSINO et al., 2009; MYROLD et al., 2013). The shotgun sequencing analysis is becoming common for environmental studies due to effectively and rapid advancement of next generation sequencing (NGS) (SCHOFIELD and SHERMAN, 2013). More specifically, metagenome sequencing comparisons between microbial communities can reveal shifts arise from natural changes (ANDREOTE et al., 2012) and anthropic variables (FIERER et al., 2012). However, there is no differentiation whether microorganisms are dead or alive in this type of analysis. For this, metatranscriptome approach is an alternative that allows identify active organisms for specific biosynthesis pathways using messenger RNA (mRNA) (BLAZEWICZ et al., 2013).

Currently, molecular tools and classical microbiological methods have been allied to provide new perspectives to create and improve bioprospection for natural products in prokaryotic organisms that are plenty of active compounds for environmental and commercial applications (NOVÁKOVÁ and FARFAŠOVSKÝ, 2013; VENTORINO et al., 2015). In this way, according to Li et al. (2014) corn field soils showed strong presence of genes related to: hemicelluloses degradation by Actinobacteria, Alphaproteobacteria and Fungi; aromatic compounds by Proteobacteria; and lignin mainly by Fungi, that could be explored for novel gene families. All information about microbes and their way of life are essential to looking for certain groups of microorganisms in appropriated places and choose specific nutrients to isolate them by culture-dependent techniques, in order to obtain uncultured or novel in appropriated places for microorganisms from the environment.

Thus, this thesis was defined to cover an evaluation of these inputs commonly applied on maize crop in Brazil and prospect microbial bioindicators through Next-Generation Sequencing (NGS) technology until allying classical microbiology isolation of active microorganisms in N_2O mitigation that would be used in new commercial bioproducts in future. Therefore, the Study I was applied to retrieve which input combination could represent better effects to reduce N_2O emissions. The Study II focused in degradation pathways of celluloses present in maize stover. And the Study III was based in modifications of culture media according to literature information and NGS prospection from previous studies in order to obtain microbial isolates with characteristics for nitrogen fixation, denitrification and cellulose degradation.

1.1 Hypotheses

The initial hypothesis was based on *Azospirillum brasiliense* characteristics that combined to other inputs can alter GHGs emissions. During the experiments and results obtained, the hypothesis evolved to three Studies. The Study I is based in test of hypothesis that the combination of commercial microbial inoculant (*A. brasiliense*) and maize stover coverage can contribute to maize development, similar to all-amendment-combined treatment, but with reduced N_2O emissions that it is increased after urea top-dressing fertilization. The Study II tested the hypothesis that cellulose decomposition is selective for microbial communities prone to use previous nitrogen forms before denitrification resulting in N_2O and/or the nitrogenase enzyme of nitrogen fixing microorganisms can act in favor of denitrifying process. Finally, in the Study III, modified culture media were tested to retrieve and isolate microorganisms under

low oxygen concentration (2% O_2) that could be capable to degrade cellulose, fixing nitrogen and/or denitrify nitrogen avoiding N_2O emissions.

1.2 Objectives

1.2.1 General objective

The objective purposed was to evaluate urea top-dressing fertilization, microbial inoculant and maize stover interactions with rhizosphere communities in Brazilian tropical soil in controlled conditions experiments, in order to identify bioindicators for nitrous oxide (N_2O) mitigation in a first study. For a second study, we aimed understand the temporal activity pathways of microbiota in celluloses decomposition in corn field soil and Amazonian Dark Earth (ADE). In continuity, the objective of the third study was to prospect and isolate microorganisms under hypoxic condition that can act in nitrogen fixation, denitrifying process and/or convert cellulose in favor of greenhouse gases (GHGs) mitigation.

The evaluation of these inputs interaction was performed through: (a) GHGs fluxes measurements through gas chromatography; (b) quantitative PCR (qPCR) for genes related to Nitrogen cycle and methane cycle; (c) 16S rRNA gene sequencing using NGS. For analysis of microbiota activities in cellulose decomposition was performed NGS based on metagenome and metatranscriptome approach.

1.2.2 Specific objectives

a) Study I: The Maize Rhizosphere Interactions – Characterization of amendments interactions under controlled conditions experiments: *(a)* monitoring nitrous oxide (N_2O) flux, measured in static round-chambers using gas chromatography (concomitantly to carbon dioxide and methane fluxes – CO_2 and CH_4, respectively) during corn plant cycle; *(b)* correlating gases emissions to genes presence through real-time quantitative PCR (qPCR) of Nitrogen cycling genes (*nifH* and *nosZ*) for nitrogen fixation and denitrifying, respectively, and methane cycling genes (*mcrA* and *pmoA*) for emission and consumption of methane, respectively, in two Stages (V5 and V15) of plant development from maize rhizosphere; and *(c)* identifying microbial populations structure of treatments in 16S rRNA gene sequencing;

b) Study II: The Degradation Process – Analyze metagenome and metatranscriptome sequencing through Illumina MiSeq System and annotate taxonomy and functions of microbiota from temporal cellulose decomposition into bulk soils with different fertility of Amazonian Dark Earth (ADE) and corn field soil (CFS), both originated from Latosols;

c) Study III: The Microbial Isolation – Prospect and isolate microorganisms from ADE and CFS of Study II under hypoxic condition (2% O_2) using modified media: nitrogen-free combined carbon (NFCC) medium, carboxymethylcellulose (CMC) medium and microcrystalline cellulose or Avicel™ (AC) medium; detect genes in isolated microorganisms for xylan degraders and Nitrogen cycling genes using conventional PCR.

1.3 Thesis structure

This thesis consists of an introductory initial text followed by three studies presented in scientific manuscript format written in English language.

1.4 INTRODUÇÃO

A cultura do milho corresponde a segunda maior produção de grãos no Brasil, somente atrás da cultura da soja (EMBRAPA, 2015), com a maior demanda para fertilizantes nitrogenados (em cerca de 1,1 milhões de toneladas) que corresponde a 27,4% do total de fertilizante nitrogenado consumido entre as plantas cultivadas no Brasil (IFA, 2017). Em 2010, os solos brasileiros destinados à agricultura liberaram cerca de 452,4 Gg de N_2O para a atmosfera (84,2% do total da emissão de N_2O), em que 35,7 e 39,5 Gg de N_2O (isto é, 10,6 e 11,8 Tg em dióxido de carbono equivalente - CO_2e) foram emitidos a partir de fertilizantes sintéticos e de resíduos agrícolas, respectivamente; um incremento de 29,9% e 35,7% em comparação com a avaliação do ano de 2005 (BRASIL, 2016). Dessa forma, esforços para entender os fluxos de gases do efeito estufa (GEEs), provenientes de várias estratégias de manejo, têm sido intensivamente estudados para reduzir suas emissões direta ou indiretamente.

Vários estudos ao redor do mundo avaliam os insumos e suas influências na cultura do milho: inoculante microbiano combinado com fertilizante nitrogenado na emissão de GEEs (CALVO et al., 2016; MÜLLER et al., 2016), fertilizante nitrogenado na emissão de GEEs e produtividade (ABALOS et al., 2016), fertilizante nitrogenado na produtividade

(CAMBOURIS et al., 2016), fertilizante nitrogenado e esterco na estrutura microbiana (PEIFFER et al., 2013), aditivos orgânicos e os GEEs (THANGARAJAN et al., 2013). Mas poucos estudos exploraram os efeitos da fertilização nitrogenada, do inoculante microbiano e dos resíduos vegetais (combinados ou não) na estrutura microbiana e correlacionar suas funções nos fluxos de GEEs e na produtividade.

Muitos fatores são responsáveis pelo desenvolvimento da planta, especialmente os fatores relacionados as raízes que são o portão principal da absorção de nutrientes. Existe uma interação complexa entre as raízes e os microrganismos com ocorrência intensa de expressão de genes na rizosfera do milho do que no solo (LI et al., 2014), principalmente quando submetido ao "efeito *priming*" que é uma mudança rápida da matéria orgânica (MO) frequentemente após a adição de insumos de fertilizante orgânico ou mineral, ou exsudação de substâncias orgânicas pelas raízes que podem desencadear a emissão de GEEs, em virtude da aceleração do metabolismo microbiano e a atividade respiratória (BINGERMAN et al., 1953; THANGARAJAN et al., 2013). As raízes das plantas podem liberar substâncias orgânicas durante seu desenvolvimento e crescimento, onde vários grupos são privilegiados e podem viver intimamente próximo à fonte de nutriente; por exemplo, as bactérias diazotróficas tem sido relatadas colonizando a superfície de raízes, em troca do desenvolvimento da planta com fixação de nitrogênio, redução do nitrato e produção de fitormônios como auxinas – como o ácido indolacético (AIA) (BOTHE et al., 1992; COSTACURTA et al., 1994; BASHAN et al., 2004). Essas interações benéficas dos processos biológicos entre os exsudatos de raízes, microrganismos e os aditivos para o solo podem criar um ambiente propício ao crescimento das plantas (BADRI e VIVANCO, 2009; THANGARAJAN et al., 2013). Por esta razão, inoculantes microbianos têm sido explorados como probióticos em interações solo-planta para promover o crescimento das plantas.

Azospirillum brasiliense é uma bactéria diazotrófica de vida livre com atividade da nitrogenase para a fixação biológica do nitrogênio, que ocorre em condições de microaerofilia, onde o nitrogênio é limitado, e tem sido utilizado em culturas gramíneas (como milho, arroz e trigo) como inoculante microbiano comercial com resultados bem-sucedidos, aumentando a produtividade e produção das plantas (EMBRAPA, 2011). Essa bactéria, que pertence à classe Alphaproteobacteria, tem a forma vibrióide com um único flagelo polar que confere motilidade, também conhecida como uma rizobactéria promotora do crescimento de plantas (RPCP) através da secreção de fitormônios (OKON, 1994; SOMERS et al., 2005; AHEMAD e KIBRET, 2014)

e possui outras atividades e interações em nível rizosférico, incluindo solubilização de fosfato, degradação de sideróforos, controle biológico de patógenos do solo (BASHAN et al., 2004).

A microbiota do solo media o funcionamento sustentável do ecossistema na ciclagem de nutrientes e outros processos biológicos (por exemplo, mineralização, decomposição e fluxo de gases do efeito estufa), porventura à estas características, a estrutura de grupos microbianos são alvo de pesquisas como indicadores biológicos para a saúde do ambiente e supressão de doenças (BRUGGEN e SEMENOV, 2000). Todavia, diferenças e mudanças nas características físico-químicas têm sido consideradas na resolução das estruturas de populações microbianas entre solos (GIRVAN et al., 2003). Para uma visão geral da microbiota, análises de meta-ômicas de qualquer fonte ambiental (como solo, água, ar, e amostras humana e animal) podem prover um perfil holístico – taxonomicamente e funcionalmente – para entender os ciclos biogeoquímicos e outros processos desempenhados pelos microrganismos, explorando vias bem conhecidas ou desconhecidas (PETROSINO et al., 2009; MYROLD et al., 2013. O sequenciamento por *shotgun* está se tornando comum para estudos ambientais por sua efetividade e o rápido avanço dos sequenciamentos de nova geração (SNG) (SCHOFIELD e SHERMAN, 2013). Mais especificamente, comparações do sequenciamento de metagenomas entre comunidades microbianas podem revelar mudanças provenientes de alterações naturais (ANDREOTE et al., 2012) e variáveis antrópicas (FIERER et al., 2012). Entretanto, não existe a distinção se os microrganismos estão vivos ou mortos. Por isso, a abordagem por metatranscriptoma é uma alternativa que permite a identificação de organismos ativos por vias específicas de biossíntese usando o RNA mensageiro (RNAm) (BLAZEWICZ et al., 2013).

Atualmente, ferramentas moleculares e métodos da microbiologia clássica têm sido aliadas na promoção de novas perspectivas em criar e melhorar a bioprospecção de produtos naturais em organismos procarióticos que são repletos de compostos ativos para o ambiente e com aplicações comerciais (NOVÁKOVÁ e FARFAŠOVSKÝ, 2013; VENTORINO et al., 2015). Desse modo, de acordo com Li et al. (2014), os solos de milhos mostraram uma forte presença de genes relacionados a degradação de celulose por Actinobacteria, Alphaproteobacteria e Fungi; a compostos aromáticos por Proteobacteria; e a lignina proncipalmente por Fungi, que podem ser explorados para novas famílias gênicas. Todas informações sobre os microrganismos e seu modo de vida são essenciais na procura em lugares apropriados de certos grupos de microrganismos e o uso de nutrientes específicos para conseguir acessá-los por técnicas dependentes de cultivo, a fim de obter microrganismos não cultivados do ambiente.

Assim, esta Tese foi defina para abordar uma avaliação desses insumos comumente utilizados na cultura do milho no Brasil e prospectar bioindicadores microbianos através da tecnologia SNG até aliar a microbiologia clássica de isolamento de novos microrganismos ativos na mitigação do N_2O que futuramente poderiam ser aplicados em novos bioprodutos comerciais. Portanto, o Estudo I foi aplicado para definir a combinação de insumos em que se obtém os melhores efeitos na redução da emissão de N_2O. O Estudo II focou nas vias de degradação da celulose presente na palhada do milho. E o Estudo III se baseou na modificação do meio de cultura de acordo com informações da literatura e da prospecção por SNG de estudos anteriores a fim de obter isolados microbianos com características para a fixação de nitrogênio desnitrificação e degradação da celulose.

1.4.1 Hipóteses

A hipótese inicial foi baseada nas características do *Azospirillum brasiliense* que combinado a outros aditivos pode alterar as emissões de GEEs. Durante os experimentos e os resultados obtidos, a hipótese evoluiu para três Estudos. O Estudo I foi baseado em testar a hipótese de que a combinação de inoculante comercial (*Azospirillum brasiliense*) e a cobertura de palhada de milho podem contribuir para o desenvolvimento da cultura do milho, com resultados similares ao tratamento completo com todos os insumos combinados, exceto pela redução da emissão de N_2O que é consequência da fertilização nitrogenada de cobertura. O Estudo II testou a hipótese que a decomposição da celulose é seletiva à comunidade microbiana propensa a usar formas de nitrogênio prévias à desnitrificação resultante ao N_2O e/ou que a enzima nitrogenase dos microrganismos fixadores de nitrogênio podem atuar em favor do processo de desnitrificação. Finalmente, em um Estudo III, os meios de cultura modificados foram testados para recuperar e isolar novos microrganismos sob baixa concentração de oxigênio (2% O_2) que podem ser capazes de degradar celulose, fixar nitrogênio e/ou desnitrificar o nitrogênio evitando a emissão de N_2O.

1.4.2 Objetivos

1.4.2.1 Objetivo geral

O objetivo proposto foi avaliar as interações da fertilização de cobertura com ureia, do inoculante microbiano e da palhada de milho com a comunidade rizosférica em solos tropicais brasileiros sob experimentos com condições controladas, a fim de identificar bioindicadores

para a mitigação do óxido nitroso (N_2O) em um primeiro estudo. Para um segundo estudo, entender as vias de atividades temporais da microbiota em decomposição de celulose em solo de cultivo de milho e em Terra Preta da Amazônia (TPA). Em continuidade, prospectar e isolar microrganismos, sob condições hipóxicas, que podem atuar na fixação do nitrogênio, no processo de desnitrificação e/ou converter a celulose em favor da mitigação dos gases do efeito estufa (GEEs)

A avaliação da interação desses insumos foi realizada através da: (a) medição do fluxo de gás; (b) PCR quantitativo (qPCR) para genes relacionados ao ciclo do nitrogênio e do metano; e do sequenciamento do (c) gene 16S rRNA usando o sequenciamento de nova geração (NGS). Para as atividades da microbiota na decomposição da celulose foi realizado o NGS baseado na abordagem do metagenoma e metatranscritoma.

1.4.2.2 Objetivos específicos

a) Estudo I: As Interações na Rizosfera do Milho – Caracterizar as interações dos aditivos sob experimento de condições controladas: *(a)* monitorando o fluxo de óxido nitroso (N_2O) medido em câmaras estáticas redondas usando a cromatografia gasosa (concomitantemente ao dióxido de carbono e ao metano – CO_2 e CH_4, respectivamente) durante o ciclo do milho; *(b)* correlacionando as emissões dos gases à presença dos genes através do PCR tempo-real quantitativo (qPCR) dos genes do ciclo do nitrogênio (*nifH* e *nosZ*) para fixação do nitrogênio e desnitrificação, respectivamente, e do ciclo do metano (*mcrA* e *pmoA*) para emissão e consumo, respectivamente, em dois estádios (V5 e V15) de desenvolvimento das plantas provenientes da rizosfera do milho; e identificando a estrutura das populações microbianas dos tratamentos por sequenciamento do gene 16S rRNA;

b) Estudo II: O Processo de Degradação – Analisar o sequenciamento do metagenoma e do metatranscriptoma através do Illumina MiSeq System e anotar a taxonomia e funções da microbiota de decomposição temporal da celulose em solos de diferentes fertilidades de Terra Preta da Amazônia (TPA) e solo de campo de milho (SCM), ambos originalmente de Latossolos;

d) Estudo III: O Isolamento Microbiano – Prospectar e isolar microrganismos de TPA e SCM do Estudo II sob condições hipóxicas (2% O_2) usando meios de cultura modificados: meio combinado de carbono livre de nitrogênio (NFCC), meio

carboximetilcelulose (CMC) e meio celulose microcristalina ou Avicel™ (AC); e detectar os genes nos microrganismos isolados para degradadores de xilano e para o ciclo do nitrogênio usando PCR convencional.

1.4.3 Estrutura da tese

A tese é constituída por um texto inicial introdutório seguido por três estudos apresentados em formato de manuscritos científicos e escritos no idioma inglês.

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2 THE MAIZE RHIZOSPHERE MICROBIOTA STRUCTURE: THE BENEFITS FROM *Azospirillum brasiliense* INOCULANT AND MAIZE STOVER COVERAGE COMBINATION

Abstract

Several inputs have been studied and documented for improving crops development and production, but most of them are focused individually or in few combinations on the effects related to corn yield, greenhouse gases (GHGs) and microbial structure profile by molecular tools. Although, no one characterized the microbial composition and prospect potential responsible for GHGs emissions from rhizosphere influence under three inputs combinations in agriculture managements that are present in most of corn field cultivated in Brazil. In this study, we evaluated the influence of maize stover coverage, topdressing urea fertilization, and microbial inoculant on soil microbial composition in maize, observing greenhouse gases emissions during maize plants development in mesocosm experiment. Gas chromatography measurements showed that treatments combination could (not only) merge individual effects of each input but can potentialize their effects when combined. Observing these effects on maize plants biomass production, treatment composed by inoculant and maize stover coverage (IS) revealed as the best cost-benefit for plant biomass production (at least 10.8% more than other two- and single-variable treatments) and GHGs mitigation (12.1% less emissions than all-amendments combination treatment – i.e. FIS – which retain similar biomass production). The 16S rRNA gene sequencing expose an expressive and significative presence (in comparison to other treatments) of classes Actinobacteria (19%) and Bacilli (10%) in IS treatment, and Thermoleophilia (16,7%) and Bacilli (11,5%) in FIS treatment, that are associated to lignocellulose degradation. This way, exploration GHGs-related pathways in cellulose degradation could be essential to check the feasibility of N_2O emissions reduction.

Keywords: Microbial ecology. Denitrification. Methane cycle. Controlled experiment. PGPR.

2.1 Introduction

Several inputs have been studied and documented for improving crops development and production, soil health quality, plant pathogen resistance and benefits against seasonal variations (THANGARAJAN et al., 2013; WANG et al., 2016; SHARMA; BALI, 2018; ABBOTT et al., 2018). Considering special attention to current inputs used in agricultural crop production: (a) the synthetic nitrogen fertilization emitted 35.7 Gg of N_2O (i.e. 10.6 Tg in CO_2e) in 2010 (BRASIL, 2016); the alternative (b) commercial microbial inoculants like plant growth-promoting rhizobacteria (PGPR) are being used as biofertilizer, phytostimulator and biocontroller (OKON; LABANDERA-GONZALEZ, 1994; BLOEMBERG; LUGTENBERG, 2001); and (c) no-tillage practices adoption have been increasing between crop managements in the three biggest producers of corn around the globe: USA, China and Brazil (HE et al., 2010; USDA, 2010; FREITAS and LANDERS, 2014) providing plant residues for soil coverage and nutrients supply.

Recently studies about corn crop managements evaluation are commonly focused only in aspects like (a) corn yield, and/or (b) greenhouse gases (GHGs) emissions (CALVO et al., 2016; CAMBOURIS et al., 2016; MÜLLER et al., 2016) as consequence of the projection of production demand towards 2050 (FAO, 2009) and global climate changes reports (IPCC, 2014); and (c) microbial structure characterization using molecular approaches (PEIFFER et al., 2013; LI et al., 2014). Although, no one characterized the microbial composition and prospect potential responsible for GHGs emissions from rhizosphere influence under these three commonly used inputs combinations. Processes at rhizosphere level are complex interactions between roots, microorganisms and animals that affects plant growth (BONKOWSKI et al., 2000) and responsible for most part of activities related to biogeochemical cycles (LI et al., 2014). In addition, according to Abalos et al. (2016), information about nitrogen fertilization management to reduce N_2O without compromising crop yields still remains incomplete and urge for results.

In this study, we evaluated the influence of maize stover coverage, topdressing urea fertilization, and microbial inoculant on soil microbial composition in corn crop, observing greenhouse gases emissions during maize plants development.

2.2 Materials and Methods

2.2.1 Experimental design and setup

A greenhouse experiment was assembled to test the influence of maize stover, urea fertilization, and microbial inoculant on soil microbial composition and greenhouse gases (GHGs) emissions. These three variables were tested isolated or combined using maize mesocosms experiment.

The mesocosms experiment was assembled in the greenhouse of Cell and Molecular Biology (CENA/USP). The 24 pots (~100L volume with 8 cm of gravel as the bottom layer for water drainage) used to assemble the mesocosms were filled with top soil (25 cm) obtained from the Anhumas Experimental Station (ESALQ/USP; 22º50'28.22" S, 48º1'1.7" W) under crop production conditions. The soil is classified as Dystrophic Red-Yellow Latosol. Dystrophic Latosols represents the major part of soils used for corn production in Brazil (IBGE, 2018; CONAB, 2018). At this point, aliquots (~300 g) were sent to Department of Soils (ESALQ/USP) for physicochemical analysis to calculate soil fertility correction – pH was determined using KCl, OM content was determined by colorimetric method; P was determined using Mehlich-1 extraction; and K, Ca and Mg were determined by ion exchange resin-based method. Thus, 08-28-16 NPK formulation were added 280 kg/ha in planting furrow during seeds sowing for fertile correction and plant health maintenance until 15th mature leaf (V15 Stage) sampling.

The maize stover was collected from Anhumas Experimental Station (ESALQ/USP) and applied as in-field conditions (500 kg/ha). Topdressing urea fertilization was applied at 5th mature leaf (V5 Stage) according to in-field conditions (90 kg/ha). The inoculant used was *Azospirillum brasiliense* – Masterfix® Gramíneas (Stoller do Brasil LTDA) – and it was applied at seeds sowing following manufacturer's instructions (100 mL/ha). A control condition including only mesocosms with plants was also considered. All the conditions were performed in triplicates and disposed in three blocks randomly (one replicate in each block).

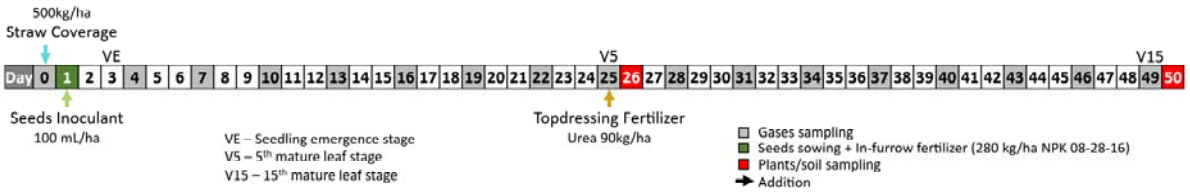
Maize plants were grown from AG 8088 seeds variety with VT Pro Yieldgard technology (Sementes Agroceres - Monsanto, Santa Cruz das Palmeiras, SP, Brazil) suitable for grain and silage production on first and second crop with high-temperatures tolerance.

The experiment was conducted for 50 days until V15 Stage of maize plants development. During the experiment, soil humidity was kept similar among treatments with daily irrigation based on water consumption of maize (EMBRAPA, 2015). Soil temperature

oscillated from 21ºC to 30ºC and air temperature varied from 21ºC to 45ºC during the day in the experiment period.

Observing the experiment timeline (Figure 1), treatments changes occur in different time. Maize stover coverage (S) and *A. brasiliense* inoculant (I) treatments start their influence in the beginning of the experiment and topdressing fertilization (F) treatments start their influence after V5 Stage with topdressing urea fertilization. It means that F treatments (F, FI, FS and FIS) have same characteristics as C, I, S, and IS treatments before V5 Stage, respectively. Consequently, these treatments would be grouped before V5 Stage, presenting similar results as four treatment pairs (C/F, I/FI, S/FS and IS/FIS).

Figure 1 – Experiment timeline



2.2.2 Gases sampling and analysis

Static round-chambers (20 cm inner diameter) were installed in center of each pot to monitor differences in gases fluxes during maize plants growth until V15 Stage. The chambers anchors were placed 5 cm depth from surface to not affect water movement in the soil and ensure gas retention in the chambers (CERRI et al., 2013).

Gases sampling was realized in the morning, between 10 AM and 12 PM, every 72 hours after seeds sowing until V15 Stage. After attach the chamber lids on chamber anchors, gases samples of 20 mL were collected in four times (1, 15, 30 and 45 minutes) and stored into plastic syringes for each chamber/pot – and load in the same day into SRI 8610C gas chromatography instrument (SRI Instruments) set to detect CH_4, CO_2 and N_2O gases, accordingly to manufacturer's operating manual. Also, an ambient air sample at initial time (T0) was collected as quality control of air samples and for gases measurement normalization, and air temperature data were collected for gas law correction (CERRI et al., 2013).

Each GHG emission count was evaluated from one day before sowing seeds until V15 Stage (50 days) of maize plants development and values were calculated and transformed to CO_2e (kg/ha).

2.2.3 Rhizosphere sampling

At V5 Stage (12 plants) and V15 Stage (6 plants), intact rhizosphere of maize plants was harvest from each pot as one sample. Then, aggregated soil was removed from roots and conditioned in 15 mL tubes with fast freezing using Liquid N_2 and stored at -80 ºC for further analysis. Also, soil samples were collected to check physicochemical characteristics at V5 Stage. Plants harvested were dried in oven at 60ºC for 5 days and weighted to evaluate plants development and biomass gains in the experiment.

2.2.4 DNA extraction

DNA extraction from 0.3 g of each of all rhizosphere samples were carried out using PowerLyzer PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), following manufacturer's protocol.

2.2.5 Quantitative PCR

Standard curves were prepared from serial dilutions containing between 10^5 and 10^0 copies of target gene of methane cycle, Nitrogen fixation and denitrification pathways obtained from DSMZ strains: *nifH* gene – *Bradyrhizobium japonicum* (DSMZ 30131), *nosZ* gene – *Paraburkholderia phymatum* (DSMZ 17167), *mcrA* gene – *Methanolinea mesofila* (DSMZ 23604) and *pmoA* gene – *Methylosinus sporium* (DSMZ 17706).

Triplicates of real-time reactions from V5 Stage and V15 Stage samples were carried out in a final volume of 10 µL containing 1X Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher Scientific, Vilnius, Lithuania), 1.0 µM of each primer of primer sets (Table 1) and 10 ng of template DNA and completed with ultrapure deionized water. Modified thermal cycling conditions were carried out as follows: an initial cycle of 95°C for 10 min; 50 cycles of 95°C for 30 s, (60ºC for *mcrA* gene, 58ºC for *pmoA* gene and 59°C for *nifH* gene) for 30 s and 72ºC for 30 s (acquisition data), then 1 cycle for melt curve at 95ºC for 15 s, the same temperature of annealing for each gene for 60 s and 95ºC for 15s. Thermal cycling conditions

to *nosZ* gene were a touchdown cycle as follows: an initial cycle of 95ºC for 10 min; 6 cycles of 95ºC for 20 s, 65ºC for 30 s (with -1ºC by cycle), 72ºC for 30s (acquisition data) followed by 45 of same cycles except for annealing temperature at 60ºC, then the cycle for melt curve at 95ºC for 15 s, 60ºC for 60 s and 95ºC for 15s. All real-time analyses were carried out with StepOnePlus Real-Time PCR System instrument (Applied Biosystems) and StepOne Software v2.3.

Table 1. *Primers* for qPCR of functional genes from Nitrogen and methane cycles

Gene	Primer	Sequence	Fragment	Reference
nifH	nifHF	5' AAAGGYGGWATCGGYAARTCCACCAC 3'	457	RÖSCH et al., 2002
	nifHR	5' TTGTTSGCSGCRTACATSGCCATCAT 3'		
*nos*Z	nosZ 2F	5' CGCRACGGCAASAAGGTSMSSGT 3'	267	HENRY et al., 2006
	nosZ 2R	5' CAKRTGCAKSGCRTGGCAGAA 3'		
*mcr*A	mlas-F (mod)	5' GGYGGTGTMGGDTTCACMCARTA 3'	469	ANGEL et al., 2012
	mcrA-R	5' CGTTCATBGCGTAGTTVGGRTAGT 3'		STEINBERG and REGAN, 2009
*pmo*A	A189F	5' GGNGACTGGGACTTCTGG 3'	472	HOLMES et al., 1995
	mb661R	5' CCGGMGCAACGTCYTTACC 3'		COSTELLO and LIDSTROM, 1999

2.2.6 16S rRNA amplicon sequencing

The 16S rRNA genes sequencing were performed to obtain taxonomic results from microbial populations influenced by treatments at V15 Stage samples. For this, PCR reactions were carried out in a final volume of 25 µL containing 1X Phusion High-Fidelity PCR Master Mix with HF Buffer (2X) (ThermoFisher Scientific, Vilnius, Lithuania), 0.2 µM of each 515F (5' GTGCCAGCMGCCGCGGTAA 3') and 806R (5' GGACTACHVGGGTWTCTAAT 3') primer set for V4 region from Earth Microbiome Project (EMP) (http://www.earthmicrobiome.org/), 1 µL template DNA and completed with ultrapure deionized water. The thermal cycling conditions were 3 min of denaturation at 95ºC, followed by 35 rounds of temperature cycling (95°C for 10 s, 50°C for 45 s, and 72°C for 20 s) and a final extension at 72°C for 7 min. All reactions were carried out with C1000 Touch™ Thermal Cycler with Dual 48/48 Fast Reaction Module (Bio-Rad). Aliquots (5 µl) of PCR products were checked on GelRed-stained 1% agarose gels in 80 V by 40 min and quantified using Qubit 2.0 following manufacturer's instructions. All PCR products were purified using QIAquick PCR

Purification Kit (QIAgen) following to manufacturer's instructions. Purified PCR products were sent to University of California Davis Genome Center Facility (Davis, CA) for sequencing on HiSeq 2500 (Illumina Inc.).

2.2.7 Computational and statistical analysis

Raw nucleotide sequences produced by forward and reverse primers for V4 region of 16S rRNA gene were analyzed using Qiime2 microbiome bioinformatics platform (https://qiime2.org). Sequences were denoised using dada2 (for quality control of sequences and chimeric sequences filtering), then rarefied to 50,000 sequences and aligned to Silva 119 release database based on 99% sequence identity as taxonomic reference.

Diversity indexes were calculated from aligned sequences using Qiime2. Non-metric multidimensional scaling (NMDS) and PCoA analysis were generated using vegan (OKSANEN et al., 2017) and ape (PARADIS et al., 2004) package and plotted using ggplot2 (WICKHAM, 2009) in R statistical environment (R CORE TEAM (2017).

Shapiro-Wilk normality test was performed in order to define the most appropriate statistical test to be used to detect significant differences among treatments. For $p<0.05$, post-hoc test for multiple comparisons were performed using Dunn's test from dunn.test package (DINNO, 2017), otherwise TukeyHSD test was implemented using agricolae package (MENDIBURU, 2017), both in R statistical environment.

The STAMP v2.1.3 (PARKS et al., 2014), a graphical software for statistical analysis of taxonomical and functional profiles was used to determine statistical differences among rhizosphere-soil treatments. The p-values were calculated using Welch's t-test two-sided with Welch's inverted as the method to calculate confidence intervals at 95%. Storey FDR ($q > 0.05$) multiple test correction was applied for effect size filter in difference between proportions (DP < 1.00).

2.3 Results

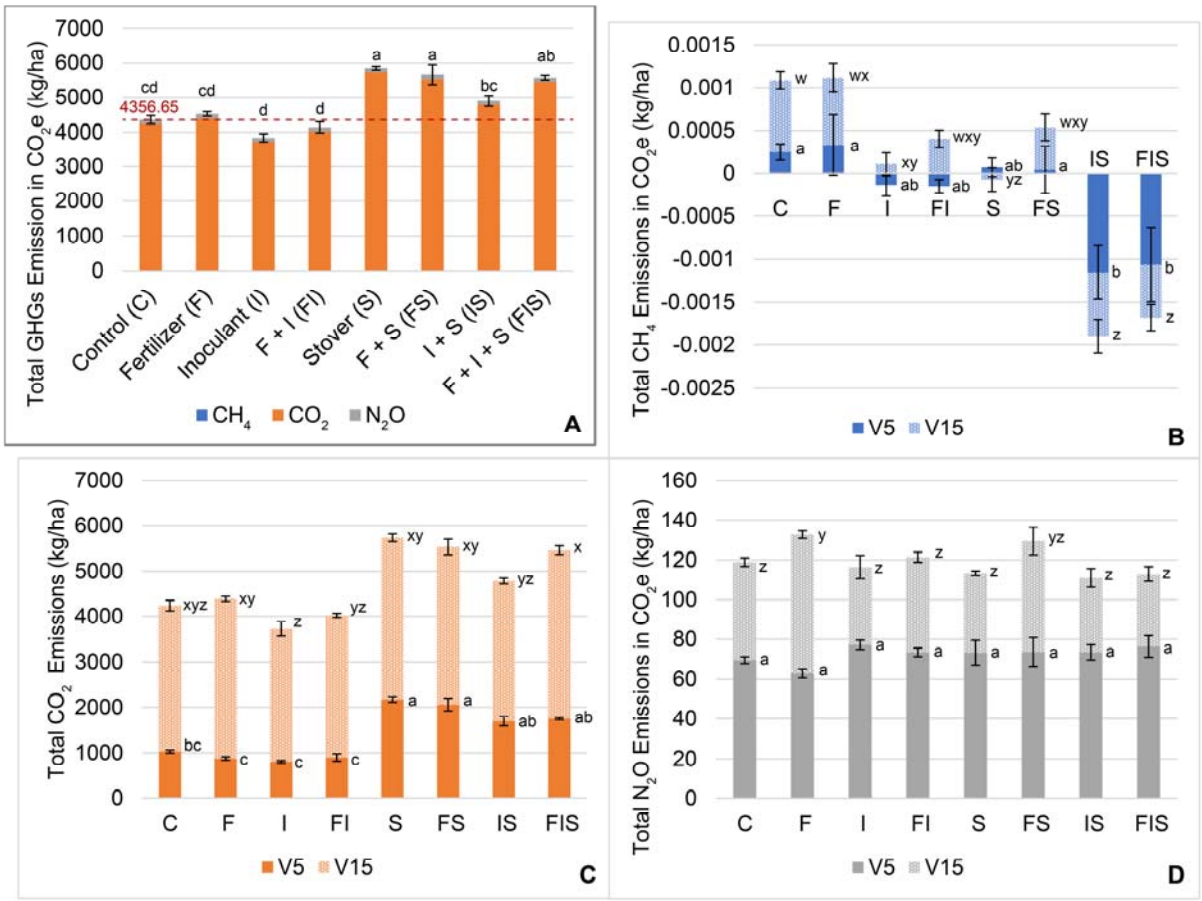
2.3.1 Physicochemical characterization

Few slight differences in soil characteristics among treatments were observed, in which pH and magnesium (Mg) values of fertilizer and maize stover combination (FS treatment) were minor in comparison to others, meanwhile maize stover (S) treatment has trends for higher values (Supplementary Table 1).

2.3.2 Gases emissions

From results of gases fluxes in CO_2 equivalent, the control group presented 4356.65±117.06 kg/ha (CO_2e) of total GHGs emissions as reference for treatments (Figure 2.A). CO_2 fluxes from soils is higher than N_2O and CH_4 fluxes in all treatments.

Figure 2 – Emissions in carbon dioxide equivalent (CO_2e) from maize soil-rhizosphere experiment: (A) all three main GHGs, (B) methane (CH_4), (C) carbon dioxide (CO_2), and (D) nitrous oxide (N_2O). Bars with same letters are not significantly different ($p < 0.05$). Dotted line in A represents total GHGs emissions from control group as reference. Graphics using two group series of letters compares treatments from each gas and stage period distinctly (abcd for V5 and wxyz for V15). V5 includes accumulated gas measurements between seeds sowing until rhizosphere sampling at V5 Stage; while V15 includes accumulated gas measurements between rhizosphere samplings (V5 and V15 Stages)



In total sum of GHGs emissions (Figure 2.A), treatments containing *A. brasiliense* inoculant (**I**/F**I**/**IS**/F**IS**) presented, respectively, 11.7%, 8.3% 16.2% and 1% of reduction on emissions comparing to other treatments without inoculant (C/F/S/FS), with IS demonstrating

the most pronouced effect of GHG mitigation ($p < 0.05$). The GHG emissions obtained from the topdressing urea fertilization treatments, compared to the other groups (i.e., **F**:C/**FI**:I/**F**S:S/**F**IS:IS) variate 4%, 8.4%, -2.8% and 14%, respectively. Finally, the treatments influenced by maize stover (i.e., **S**/F**S**/I**S**/FI**S**) strongly increased the GHG emissions by 34,4%, 25.2%, 28.4% and 34.6% comparing the other treatments (i.e., C/F/I/FI).

Analyzing each GHGs (Figure 2.B, C and D), treatments I-FI presented tendency to CH_4 consumption before V5 Stage, while maize stover treatment (S) presented this trend after V5 Stage. These trends from both I-FI and S treatments were potentialized and become significantly when combined in IS and FIS treatments; before and after V5 Stage (Figure 2.B).

In CO_2 measurements (Figure 2.C), S treatments are related to this gas emissions increments at V5 Stage, however I treatments presented reduced emissions after V5 Stage in comparison to other inputs treatments.

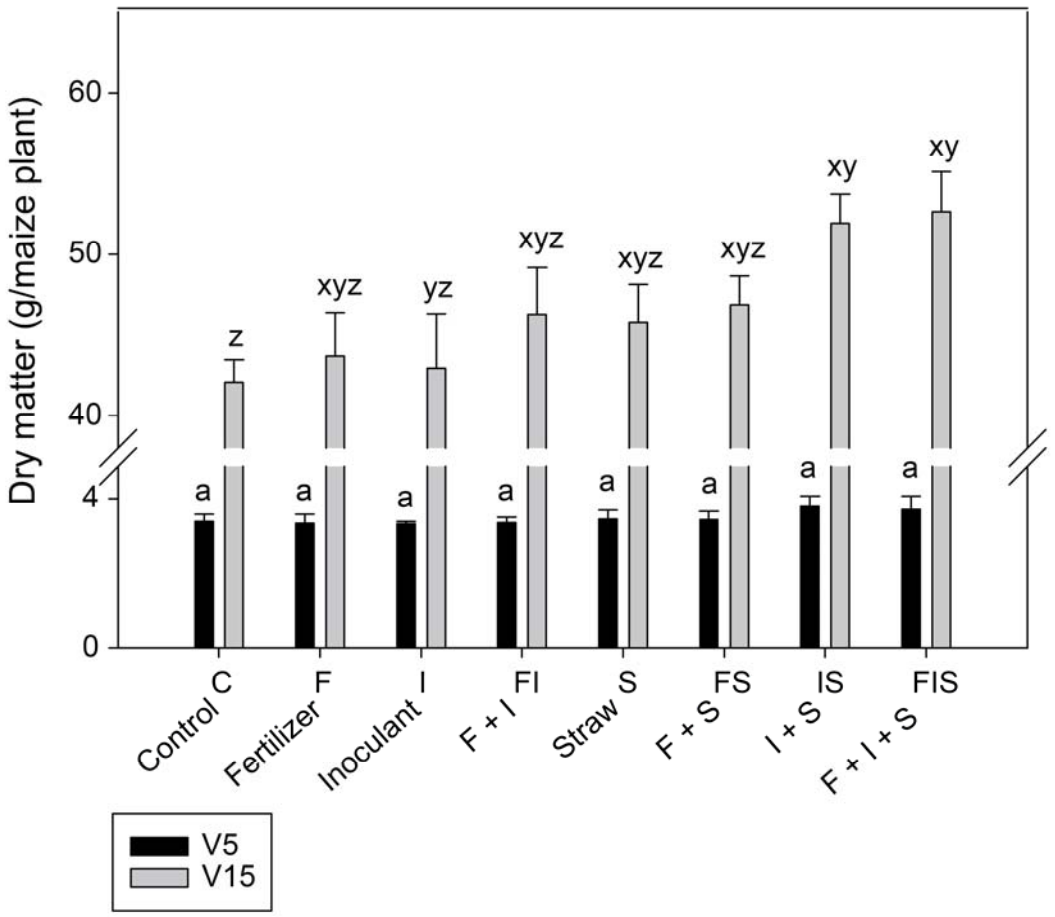
Topdressing urea fertilizer (after V5 Stage) has increased N_2O emissions significantly in F treatment ($p < 0.01$) and presented a slightly tendecy in FS, circa 21.9~39.2% of N_2O increment after application (comparisons **F**:C/**FI**:I/**FS**:S) except for **F**IS:IS with only 2.5% (Figure 2.D).

2.3.3 Dry matter of maize plants

At V5 Stage, IS and FIS treatments (similar treatments before topdressing urea fertilization) presented a slight gaining of weight in dry matter weight average of maize plants ($n=12$; $p < 0.05$). However, at V15 Stage, IS and FIS treatments were the treatments with significantly plant biomass (23.4% and 25.4% more than C, respectively) (Figure 3).

In terms of in-field proportion with 60,000 plants/ha (the same proportion in the experiment), the plants biomass would reach 3.12 tons and 3.16 tons in dry matter of IS and FIS treatments, that means increment of 0.6 t and 0.64 tons, respectively.

Figure 3 – Dry matter average of maize plants collected in V5 and V15 Stage soil-rhizosphere sampling. Topdressing urea-fertilizer (F) at 5th mature leaf Stage (V5), seed inoculant *Azospirillum brasiliense* (I) applied at seed sowing, and maize straw coverage (S) at experiment setup and their combinations (FI, FS, IS and FIS). Bars with same letters are not significantly different ($p < 0.05$). Two group series of letters compares treatments from stage period distinctly (ab for V5 and xyz for V15)



2.3.4 Quantitative PCR

At V5 Stage, means of number of copies/µL of extracted DNA ranged: 1.86-13.06 for *mcrA* gene*;* 0.90-1.72 for *pmoA* gene; 5.87-13.25 for *nifH* gene; and 0.94-9.56 for *nosZ* gene. In comparison to V15 Stage with: 0.14-0.45 for *mcrA* gene*;* 0.88-2.48 for *pmoA* gene; 0.89-5.09 for *nifH* gene; and 1.92-1.72 for *nosZ* gene (Supplementary Figure 1).

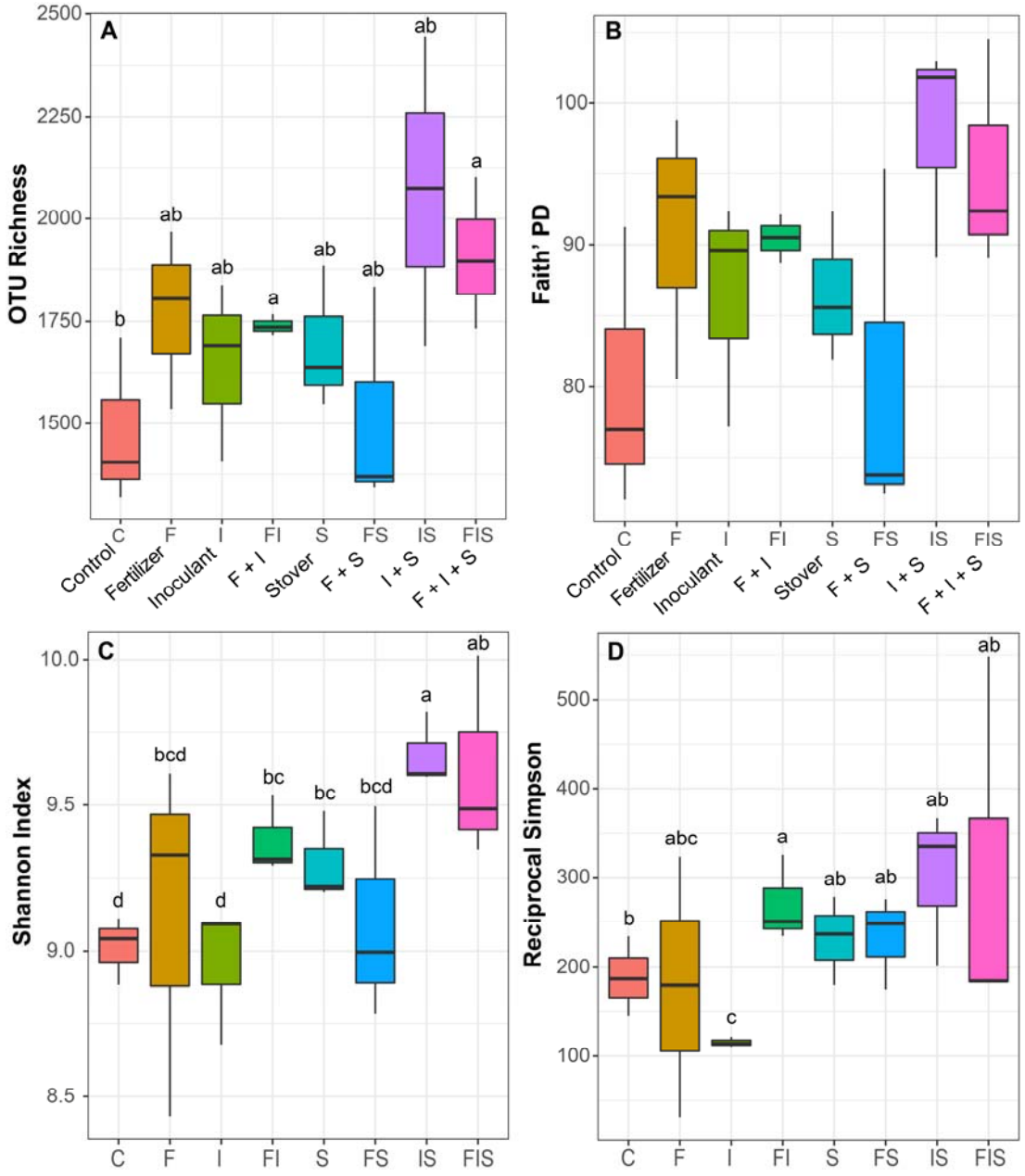
In general, S treatments (S/FS/IS/FIS) contain more copies of *mcrA* (above 10 copies/µL) genes than others at V5 Stage and *pmoA* (above 1.5 copies/ µL) at V15 Stage. Although, all treatments presented lower copies (below 1 copy/µL) for *mcrA* gene at V15 Stage.

No correlation was found among genes quantities variation. Only differences between sampling time (V5 and V15 Stage) for most of genes among treatments were observed.

2.3.5 Alpha and Beta Diversity Analyses

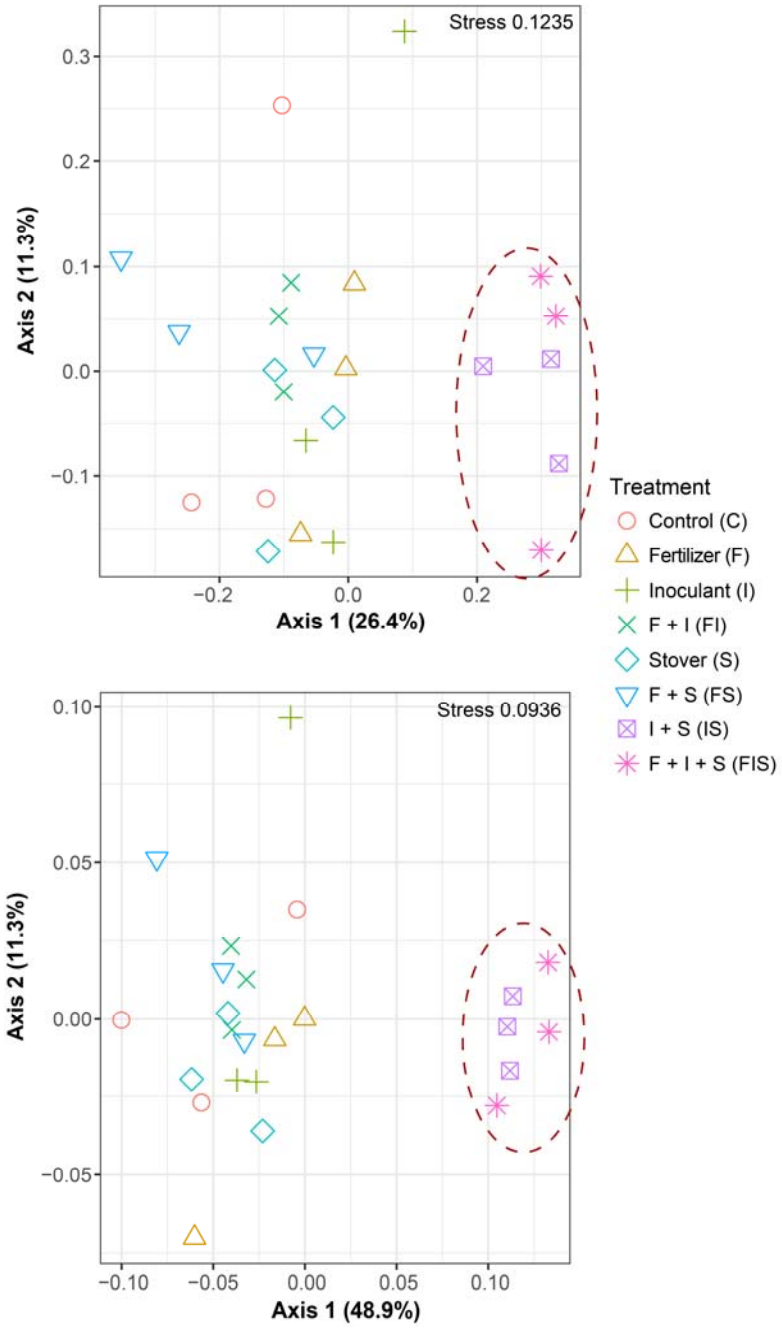
Sampling effort (50,000 sequences) was evaluated through rarefaction curves (Supplementary Figure 2). The number of OTUs reached an asymptote for all samples. Alpha diversity indexes presented a considerable increase for IS and FIS in multiple comparisons among treatments, but not diverge statistically (Figure 4).

Figure 4 – Alpha diversity boxplots from V15 Stages of maize rhizosphere sampling of (A) total species richness, (B) Faith's phylogenetic diversity, (C) Shannon index, and (D) Reciprocal Simpson index (1/D). Topdressing urea-fertilizer (F) at 5th mature leaf Stage (V5), seed inoculant *Azospirillum brasiliense* (I) applied at seed sowing, and maize stover coverage (S) at experiment setup and their combinations (FI, FS, IS and FIS)



Although, taxonomic (Bray-Curtis - $R = 0.4157$, $P < 0.002$) and phylogenetic (weighted UniFrac - $R = 0.461$, $P < 0.001$) approaches were used to estimate community dissimilarities and beta diversity among rhizosphere-soil samples (Figure 5). IS and FIS treatments are grouped distinctly from all other treatments.

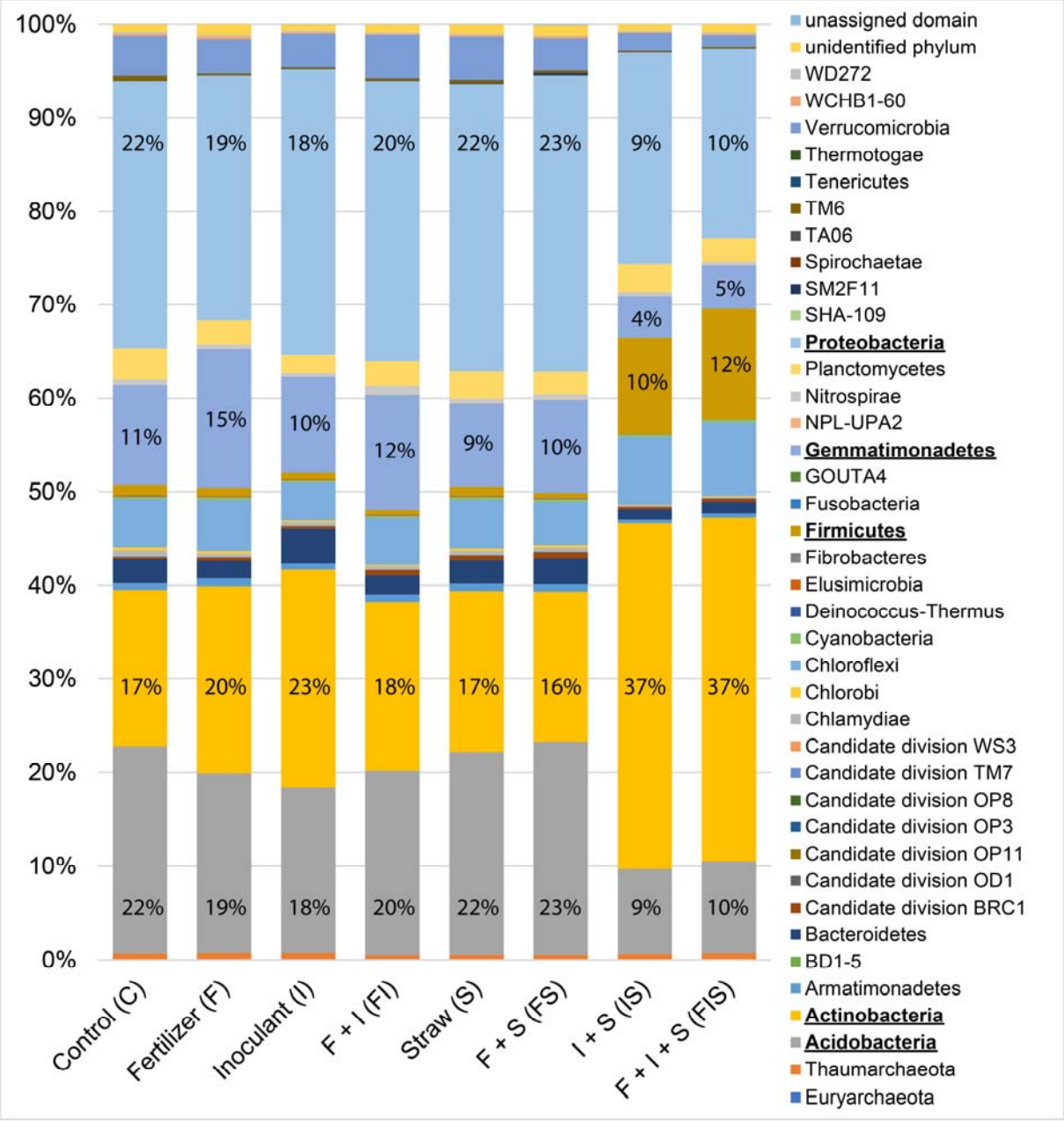
Figure 5 – Non-metric multidimensional distance scaling (NMDS) analysis of community composition of rhizosphere among treatments at V15 Stage. (A) taxonomic similarity (Bray-Curtis) and (B) phylogenetic similarity (weighted UniFrac). Topdressing urea-fertilizer (F) at 5th mature leaf Stage (V5), seed inoculant *Azospirillum brasiliense* (I) applied at seed sowing, and corn-straw coverage (S) at experiment setup and their combinations (FI, FS, IS and FIS)



2.3.6 Microbial communities structure

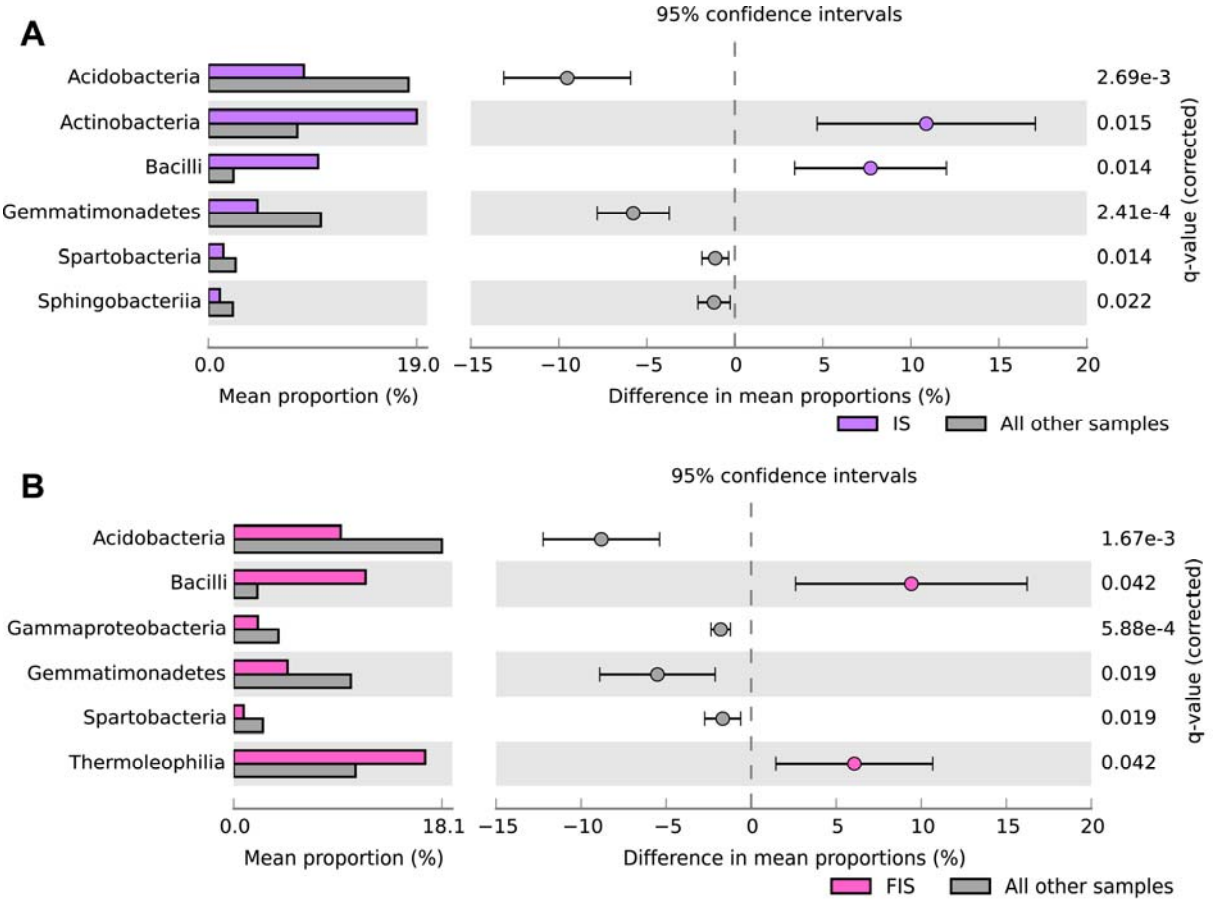
From all 400,000 sequences analyzed, a total of 10,497 OTUs were identified and distributed in 39 phyla with 0.7~1.1% of unidentified phylum using Silva 119 99% database (Figure 6).

Figure 6 – Relative abundance of taxonomic affiliation from 16S rRNA genes of maize rhizosphere under different managements at V15 Stage. Topdressing urea-fertilizer (F) at 5th mature leaf Stage (V5), seed inoculant *Azospirillum brasiliense* (I) applied at seed sowing, and corn-straw coverage (S) at experiment setup and their combinations (FI, FS, IS and FIS). Sequences were aligned against Silva 119 99% dataset using Qiime2 software platform.



Significant differences at class level were observed for IS and FIS compared to other treatments. For IS treatment, presented Actinobacteria (19%), Bacilli (10%), Acidobacteria (8.7%), Gemmatimonadetes (4.5%), Spartobacteria (1.4%) and Sphingobacteria (1%) in relative abundance (Figure 7.A). While FIS treatment presented Thermoleophilia (16.7%), Acidobacteria (9.3%), Bacilli (11.5%), Gemmatimonadetes (4.7%), Gammaproteobacteria (2.1%) and Spartobacteria (0.9%) as significant classes in relative abundances differences (Figure 7.B).

Figure 7 – Statistical difference of representative groups of Class level at V15 Stage rhizosphere samples. **A -** Inoculant + Straw (IS) against all other treatments; **B –** Fertilizer + Inoculant + Straw (FIS) against all other treatments. Error bars are standard deviation. q-values were calculated using Storey FDR approach ($p < 0.05$) with effect size filter in difference between proportions (DP < 1.00)



2.4 Discussion

A brasiliense as known as plant growth-promoting rhizobacteria (PGPR) and diazotrophic bacteria, also retain nitrogenase activity for biological N_2 fixation (SOMERS et al., 2005; AHEMAD; KIBRET, 2014) and has other activities at the rhizosphere interactions level including phosphate solubilization, degradation of siderophores, biological control of soil-borne pathogens (BASHAN et al., 2004). Treatments containing *A. brasiliense* inoculant showed reduction on CH_4 and CO_2 emissions during corn crop and increase maize plant biomass yield and stimulates roots development. This plant stimulation could lead to better root development on initial stages and prepare to open-wide nutrient intake system for subsequent reproductive stages (EMBRAPA, 2015; VACHERON et al., 2013). In this way, getting bigger plants allow more CO_2 consumption and more biomass increments.

Furthermore, diazotrophic bacteria have been related not only to nitrogen fixation, but also being capable of reducing nitrate and producing phytohormones like auxins – e.g. indole-3-acetic acid (IAA) (BOTHE et al., 1992; COSTACURTA; KEIJERS; VANDERLEYDEN, 1994; BASHAN et al., 2004). Moreover, the nitrogenase enzymatic system formed by ferro-molybdenium (Fe-Mo) has similar ligation sites can reduce both N_2 and N_2O, showing close relations between N_2-fixing and denitrifying bacteria (MOZEN; BURRIS, 1954).

Besides microbial inoculants, maize stover presence (S treatments) stimulate a vigorous development of maize plant and act as shelter of other microorganisms that arise dominant. On the other hand, maize stover decomposition seems like to be the main responsible to increase CO_2 emissions during the experiment.

In other study, Peiffer et al (2013) obtained several core-dominant microbial phyla structure affiliations using same set of primer in different condition of maize plant variety and climatic region. Comparing to our microbial profile composition of rhizosphere, these similarities could imply in common structure of rhizosphere with most important microbial communities for maize plants.

Functionally, maize rhizosphere commonly selects groups from bulk soil that act on carbon fixation and carbon degradation pathways, including exopolysaccharides and antibiotic producers to control soil-borne pathogens (LI et al., 2014). These functional groups are expected due to large capacity of exudate composition and production from maize roots (CARVALHAIS et al., 2011). The bacterial groups that distinguished IS and FIS treatments from others would act indirectly in rhizosphere structure due to their influence as tenacious

substrate competitors and antimicrobial producers, also they have the ability of sporulation. These characteristics could become them as persistent in environment like some classes as Actinobacteria and Bacilli. Actinobacteria are known as antibiotic producers, saprophytic, and plant growth-promoting rhizobacteria (PGPR) (DOUMBOU et al 2001). Bacilli are PGPR, cellulose and hemicellulose degraders, biosurfactants and carotenoids pigments producers, and act as biopesticides (LEE et al., 2008; DI PASQUA et al., 2014). However, IS and FIS treatments loses abundance in Acidobacteria that are versatile in carbohydrate metabolism and use nitrite as N source (KIELAK et al, 2016), that could be explained by competition for nitrite intake against corn plants with higher biomass.

Overall, the three variables evaluated in this paper have shown particular major responses in single-variable treatments: (a) topdressing urea-fertilizer increase N_2O emissions around 1-week effect after application, as also Calvo et al. (2016) reported; (b) *A. brasiliense* inoculant induced reduction on CH_4 and CO_2 emissions; (c) maize stover coverage shown an increment for CO_2 emissions.

On the other hand, two- and three-variable interactions potentialized or merged single-variable responses: (a) FI treatment shown mid-values of total CO_2 and N_2O emissions between F and I treatments; (b) FS treatment retained similar N_2O and CO_2 emissions from F and S treatments, respectively; (c) FIS treatment merged patterns of GHG emissions from F, I and S treatments, except for CH_4 that potentialized I and S responses for reduction of emission, with advantage of higher levels of dry mass gains; and (d) IS treatment shown similar to FIS treatment with inoculant influence on reduction of CO_2 emissions that rises as best cost-benefit treatment for higher production with less GHG emissions.

2.5 Conclusion

In conclusion, this study traced resolution of inputs effects on soil microbiota and evaluated their contribution to corn crop development on greenhouse gases (GHGs) fluxes. As main result, the combination uses of microbial inoculant and maize stover coverage is the best option, aiming for high biomass production of maize plants with beneficial reduction of GHGs emissions. Additionally, the taxonomic profile stablished an enrichment of classes (Actinobacteria and Bacilli), in IS and FIS treatment, that are associated to cellulose degradation. This way, exploration GHGs-related pathways on cellulose degradation could be essential to check the feasibility of GHGs emissions reduction.

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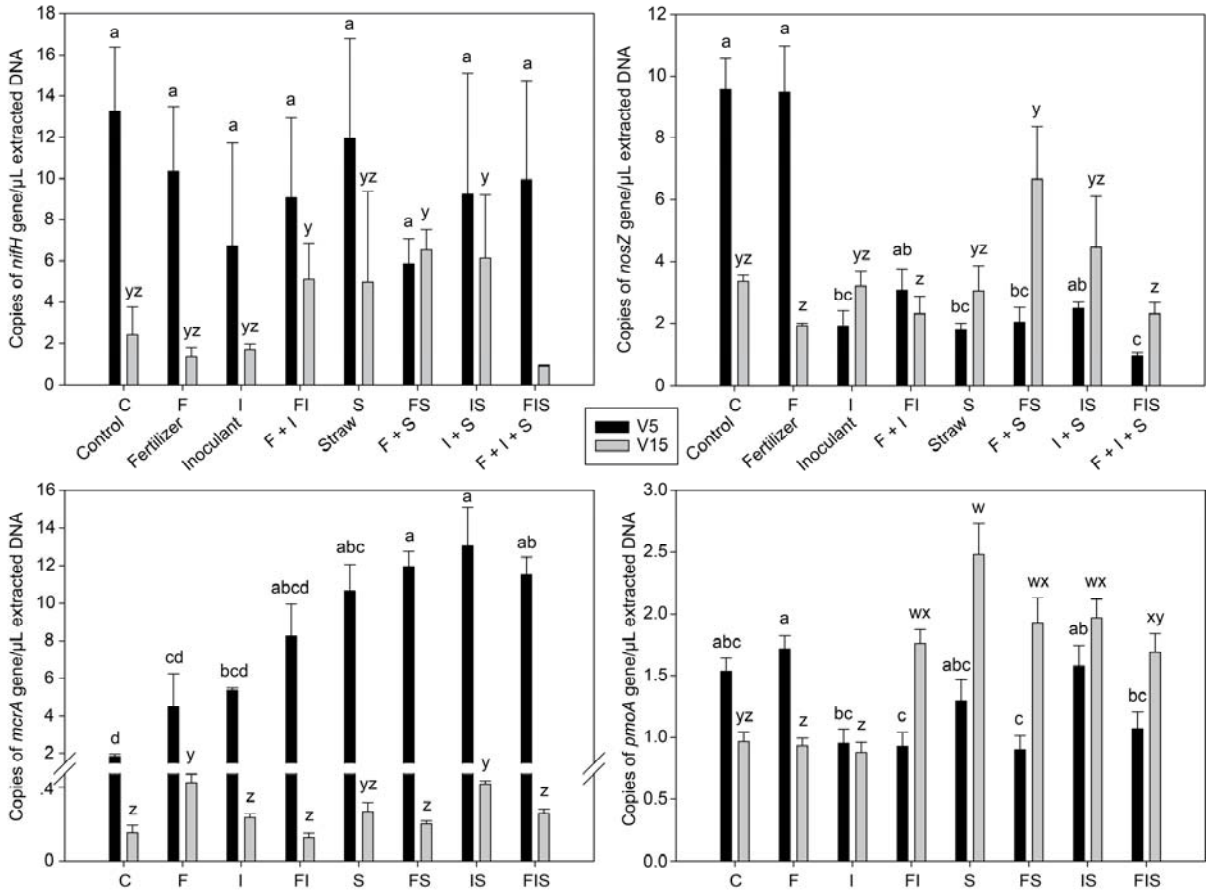
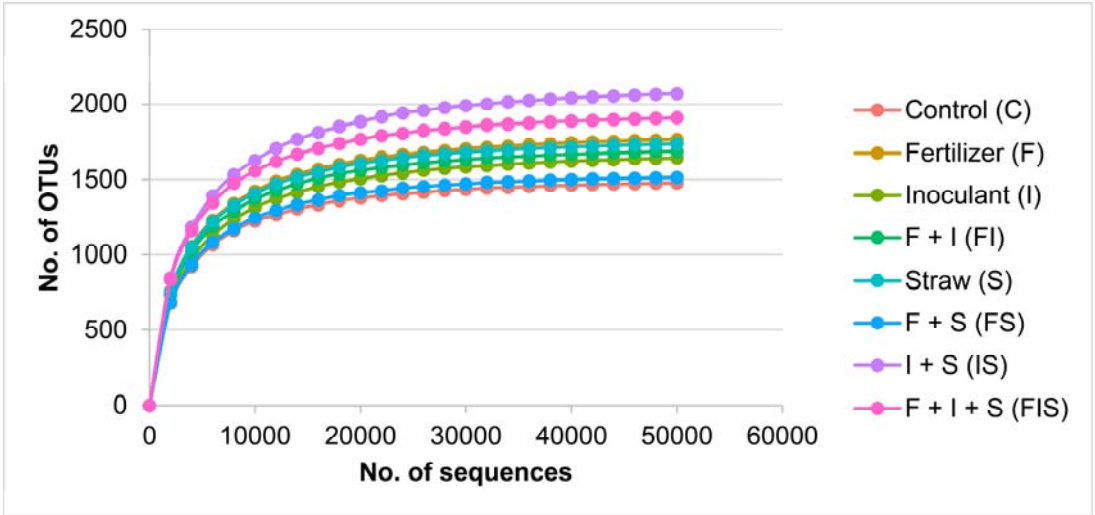
Supplementary Figure 1 – Quantitative PCR of Nitrogen (A and B) and methane (C and D) cycles genes at maize rhizosphere sampling (V5 and V15 Stages). Topdressing urea-fertilizer (F), seed inoculant *Azospirillum brasiliense* (I), and maize stover coverage (S) and their combinations (FI, FS, IS and FIS). Bars with same letters are not significantly different ($p < 0.05$). Graphics with two bars are compared using two group series of letters (abcd and wxyz).



Supplementary Figure 2 – Rarefaction curve of total OTUs obtained from all treatments of maize rhizosphere at V15 Stage: Control (C), Topdressing urea fertilizer (F), *Azospirilum brasiliense* inoculant (I), maize stover coverage (S) and their combinations (FI, FS, IS and FIS)



Supplementary Table 1 – Average soil chemical characteristics of treatments at V5 Stage. Values are mean ±SE, n = 3. Columns mean followed by same letter or letter absence are not significantly different ($p < 0.05$) bases on Tutkey HSD test and Dunn test for normal and non-normal values, respectively. Ca, Mg, Al, potencial acidity (H+Al), sum of bases (SB) and cation exchange capacity (CEC) are expressed in $mmolc.kg^{-1}$. P is expressed in $mg.kg^{-1}$. Organic matter (OM) is expressed in $g.kg^{-1}$.

Treatment	pH	CEC	V	m	OM	P	K	Ca	Mg	Al	H+Al	SB
Control (C)	6.2±0.1 a	43.3±0.9	60.7±3.2	0.0	8.7±1.5	41.0±1.0	3.0±0.6	18.7±1.2	5.0±0.0 ab	<1	16.7±1.2 ab	26.7±1.8
Fertilizer (F)	6.2±0.1 a	48.0±1.5	60.3±2.2	0.0	9.0±3.0	39.7±0.3	3.3±0.7	21.0±1.2	5.0±0.0 ab	<1	18.7±0.9 ab	29.3±1.8
Inoculant (I)	6.1±0.1 a	44.3±1.7	58.7±3.0	0.0	7.3±0.7	38.3±1.2	3.0±0.6	18.7±1.5	4.7±0.3 ab	<1	18.0±0.6 ab	26.3±2.2
F + I (FI)	6.1±0.1 a	43.0±2.9	56.3±4.1	0.0	8.3±1.2	37.0±0.6	2.3±0.3	17.3±2.6	5.0±0.6 ab	<1	18.3±0.7 a	24.7±3.5
Straw (S)	6.3±0.1 a	47.7±0.3	61.7±2.2	0.0	8.0±1.5	38.3±1.9	3.0±0.6	20.7±1.2	6.0±0.0 a	<1	18.0±1.0 ab	29.7±1.2
F + S (FS)	5.6±0.1 b	43.3±0.7	52.0±2.0	0.0	7.7±2.7	38.3±1.3	2.3±0.3	16.0±1.0	4.3±0.3 b	<1	20.7±0.9 ab	22.7±1.2
I + S (IS)	6.1±0.1 a	43.3±0.3	56.3±3.2	0.0	8.7±0.7	37.7±0.7	2.3±0.9	17.3±0.7	5.0±0.0 ab	<1	18.7±1.5 ab	24.7±1.5
F + I + S (FIS)	6.1±0.1 a	42.0±3.1	60.7±2.3	0.0	7.7±0.3	42.7±5.2	3.7±2.2	16.7±0.7	5.3±0.3 ab	<1	16.3±1.8 b	25.7±1.8

3 METATRANSCRIPTOME ASSESSMENT FOR PLANT RESIDUE DEGRADATION IN TWO BRAZILIAN LATOSOLS: A FOCUS ON METHANE AND NITROUS OXIDE FUNCTIONS

Abstract

Culture-independent high-throughput metatranscriptome sequencing is important to reveal uncultivated microbiota niches and explore ecological and taxonomic diversity at high resolution. This tool allows the searching for potential sources of novel enzymes and can be more sensitive to environmental variations than metagenome approaches. In this study, we propose a particular evaluation of enzymes expression related to nitrogen and methane metabolisms with taxonomic and functional characterization of active microbiota on maize stover degradation under two Brazilian latosols with distinct fertility properties for comparative proposes. In order to magnify pathways exclusively to lignocellulose process, nucleic acids extractions were realized directly from degraded maize stover and its adhered soil, concomitantly, this methodology allows to avoid, or at least reduce, contaminants and library construction inhibitors that are present in soil as acids, furan derivatives and phenolic compounds. Metagenome and metatranscriptome were sequenced from samples collected at 0, 20, 50 and 80 days after experiment setup of degradation process and analyzed on MG-RAST web server. As results, Amazonian Dark Earth (ADE) and corn field soils (CFS) has similar structure and functional profiles in metagenome approach, although differences overtime indicates that, inclusive for functions related to nitrous oxide and methane metabolisms; ADE is faster responsive for cellulose addition than CFS and the degradation process is notoriously performed by Fungi (51.3% ~ 97.8%). Apparently, few or none correlation were found between nitrous oxide reductases and nitrogen fixing pathways, but nitrite reductase and nitrate and nitrite ammonification were overwhelming expressed (3-fold) as avoiding pathway for potential nitrogen assimilation by plants. The enzymes involved in cellulose decomposition are not responsible for expressive increments on N_2O emissions, although we cannot underestimate the existence of denitrification pathway to N_2O formation. By the way, positive results from metagenome and metatranscriptome prospection directed the culture media modification to isolate microorganisms.

Keywords: Bioinformatic. NGS. Denitrification. Methane cycle.

3.1 Introduction

Organic amendments in soils generate complex ecosystem that associated to environmental conditions can stimulate a selective pressure over the biodiversity and the activity of microorganisms on biogeochemical cycles, commonly increasing greenhouse gases (GHGs) emissions during the "priming effect", as consequence of organic material input (THANGARAJAN et al., 2013). The plant residues are included as GHGs emissions stimulant in soils, although our previous results (Study I) showed the neutrality of cellulose degradation process on CH_4 and N_2O emissions – i.e. cellulose has a balanced result with same sum of emission and consumption rates of CH_4 and N_2O gases or there is no production from both at all – and reduced overall GHG fluxes when combined with *Azospirillum brasiliense* inoculant in corn crop that reflect into microbial shifts for determined phyla (*Actinobacteria* and *Firmicutes*) at V15 Stage. Nitrogen-fixing bacteria like *A. brasiliense* can interact positively to provide biological nitrogen and auxins to promoting plant growth and enlarge nutrients assimilation by plants; this higher usage of nutrients would deplete nitrogen and carbon forms to produce CH_4 and N_2O in soil and consequently mitigate these gases fluxes, effect observed in the Study I. *Actinobacteria* and *Firmicutes* were previously associated to lignocellulose degradation and also act in plant benefits for phosphate solubilization, phytohormones production and antibiotics against soil-borne pathogens (WU and HE, 2013; CASTILLO et al., 2013).

To elucidate this impasse of cellulose neutrality on GHGs, culture-independent high-throughput sequencing is being used to reveal uncultivated microbiota niches and explore ecological and taxonomic diversity at high resolution, providing a holistic profile (PETROSINO et al. 2009; MYROLD et al., 2013). This evaluation allows the searching for potential sources of novel enzymes and activities (DUAN et al. 2010; THOMPSON et al., 2013; VENTORINO et al., 2015). Recently, metagenome for characterization of biomass degradation is being well documented due to high demand for researches focused in enzymes to biofuels productions. More specifically, metatranscriptome analysis can reveal active organisms for specific biosynthesis pathways and can be more sensitive to environmental variations than metagenome approaches (BLAZEWICZ et al., 2013). Although, the metatranscriptome sequencing study is challenging due to the lack of established reference genomes, computational tools and pipelines; needing caution when interpreting and perform downstream specific analysis (JIANG et al., 2016). Despite the increase of studies in this field, most of them

make taxonomic and functional characterization of cellulose-degrading microbiota from input effects in complex soil samples instead of more exclusively approach on cellulose process itself.

In this study, we propose a particular evaluation of enzymes expression related to methane and denitrifying metabolisms with taxonomic and functional characterization of active microbiota on maize stover degradation under two latosols with different fertility for comparative proposes. In order to magnify pathways exclusively to cellulose process, nucleic acids extractions were realized directly from degraded maize stover and its adhered soil, concomitantly this methodology allows to avoid, or at least reduce, contaminants and library construction inhibitors that are present in soil as acids, furan derivatives and phenolic compounds (BATISTA-GARCÍA et al, 2016).

3.2 Materials and Methods

3.2.1 Experimental design and sampling

A mesocosm experiment was assembled to explore functional and taxonomical characterization of microbial communities responsible for maize stover decomposition that could be responsible for methane (CH_4) and nitrous oxide (N_2O) mitigation into two tropical soils.

The bulk-rhizosphere soil from corn field (CFS) (22º50'28.22"S, 48º1'1.7"W) was collected from top soil (25 cm) in the Anhumas Experimental Station of the Department of Genetics, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ) – University of São Paulo (USP). The soil is classified as Dystrophic Red-Yellow Latosol. Dystrophic Latosols represents the major part of soils used for maize production in Brazil (IBGE, 2018; CONAB, 2018). And bulk soil from Amazonian Dark Earth (ADE) was collected from top soil (25 cm) of Hatahara site (03º16.494'S60º12.340'W) – a anthropic soil, originally a Dystrophic Yellow Latosol (IBGE, 2018). ADE was transported on ice within 2 days to Cell and Molecular Biology Laboratory (CENA/USP), while CFS was promptly transported and used to execute the degradation experiment. At this point, aliquots (~300 g) were sent to Department of Soils (ESALQ/USP) for physicochemical analysis – pH was determined using KCl, organic matter (OM) content was determined by colorimetric method; P was determined using Mehlich-1 extraction; and K, Ca and Mg were determined by ion exchange resin-based method.

Pots (10L volume) were filled with collected soils and maintained in controlled conditions of temperature and moisture (25 ºC and 80% of water-filled pore space - WFPS).

Soil samples were collected at the day 0 as control group before maize stover addition. Three litterbags containing 5 g of dried maize stover were buried into each pot and sampled after 20, 50 and 80 days. One litterbag was removed for each sampling time. Litterbags samples of degraded maize stover with adhered soil were macerated to fine powder in a mortar using liquid N_2. All samples were stored in -80 ºC until nucleic acid extractions.

3.2.2 RNA extraction, mRNA enrichment and DNA extraction

RNA extraction from 1 g of each sample were carried out using RNA PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following manufacturer's protocol. The total RNA extracted was treated with dsDNase (ThermoFisher Scientific, Vilnius, Lithuania) and mRNA was isolated using Ribominus Transcriptome Isolation Kit, bacteria (Invitrogen, Carlsbad, CA). Double-stranded cDNA was synthetized using a two-step procedure: (I) SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) and (II) Mix containing DNA Polymerase I, RNase H, E. coli DNA Ligase (ThermoFisher Scientific, Vilnius, Lithuania), accordingly to manufacturer's instructions.

In order to identify microorganisms and pathways, and correlate with metatranscriptome analysis results, DNA extractions of soil samples were performed using the PowerLyzer PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), following manufacturer's protocol.

3.2.3 Library preparation and shotgun sequencing

For metatranscriptome sequencing, double stranded cDNA library was prepared using Nextera XT DNA Sample Preparation Kit (Illumina Inc., San Diego, CA). For metagenome sequencing from DNA library was prepared using Nextera DNA Sample Kit (Illumina Inc., San Diego, CA). Both libraries were sequenced with MiSeq Sequencing System (Illumina Inc., San Diego, CA) using MiSeq Reagent Kit v3 (600 cycle) (Illumina Inc., San Diego, CA), following manufacturer's instructions.

3.2.4 Metatranscritome and metagenome annotation

Sequences obtained from MiSeq Sequencing System (Illumina Inc.) from metatranscriptome dataset – Read 1 (R1) and Read 2 (R2) – were submitted to the metagenomics RAST Server (MG-RAST version 4.0.3 - http://metagenomics.anl.gov/index.html; MEYER et al. 2008), a commonly used web server

pipeline for metagenome and metatranscriptome annotation for downstream analysis. Taxonomic and functional profiles were generated using normalized sequences matches to RefSeq and SEED database, respectively, and the frequency of representative hits were normalized dividing by the total number of hits of each sample.

3.2.5 Statistical analysis

Diversity indexes and non-metric multidimensional distance scaling (NMDS) were calculated using vegan (OKSANEN et al., 2017), and plots were generated using ggplot2 (WICKHAM, 2009) in R statistical environment (R CORE TEAM, 2017).

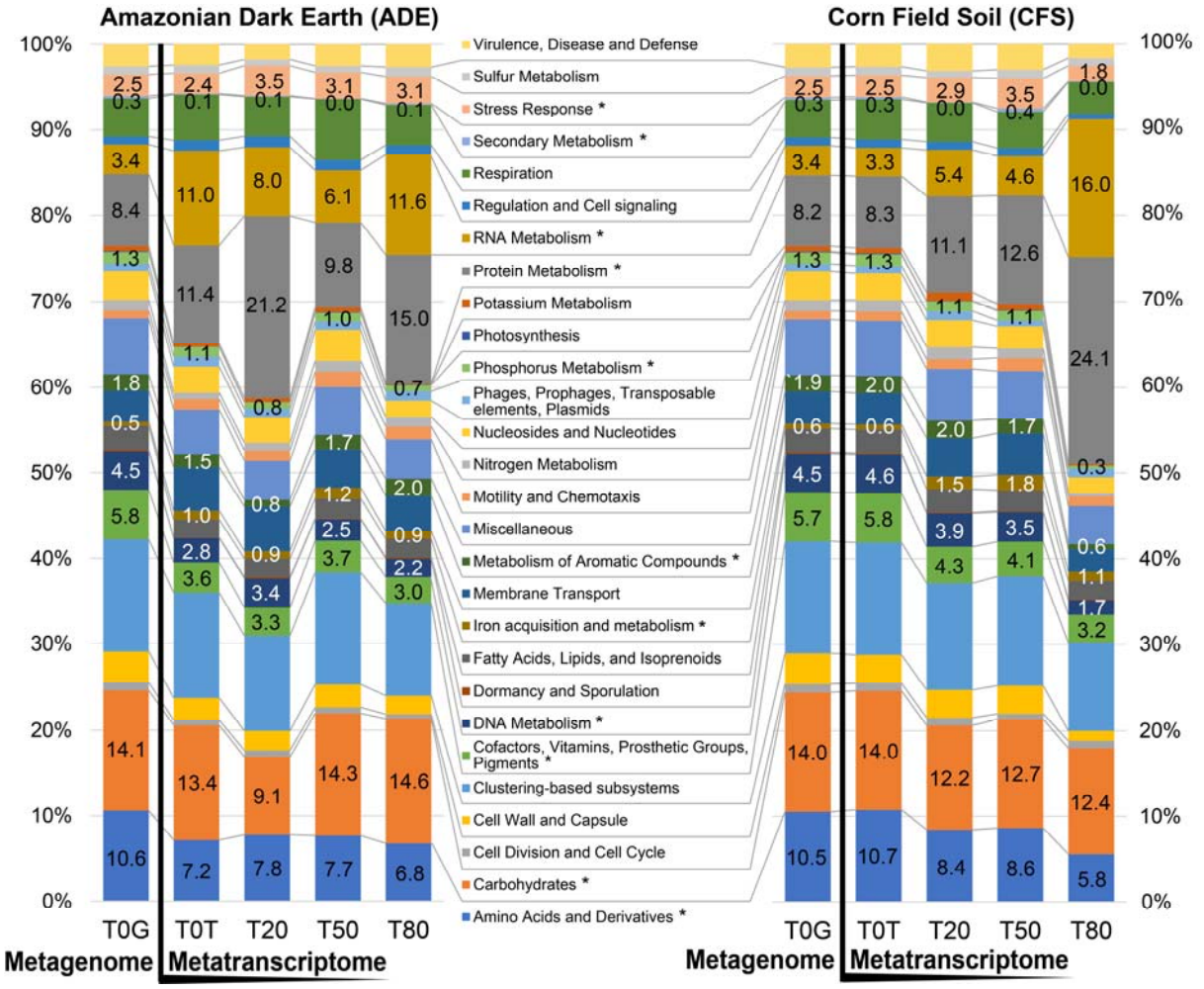
The STAMP v2.1.3 (PARKS et al., 2014), a graphical software for statistical analysis of taxonomical and functional profiles was used to determine statistical differences among rhizosphere-soil treatments. The p-values were calculated using Welch's t-test two-sided with Welch's inverted as the method to calculate confidence intervals at 95%. Multiple test corrections were applied which best suits for p-value correction, considering Storey FDR ($q > 0.05$) for dependency among time and the more conservative correction of Benjamin-Hochberg ($q > 0.05$) for soil comparisons.

3.3 Results

3.3.1 Functional approach

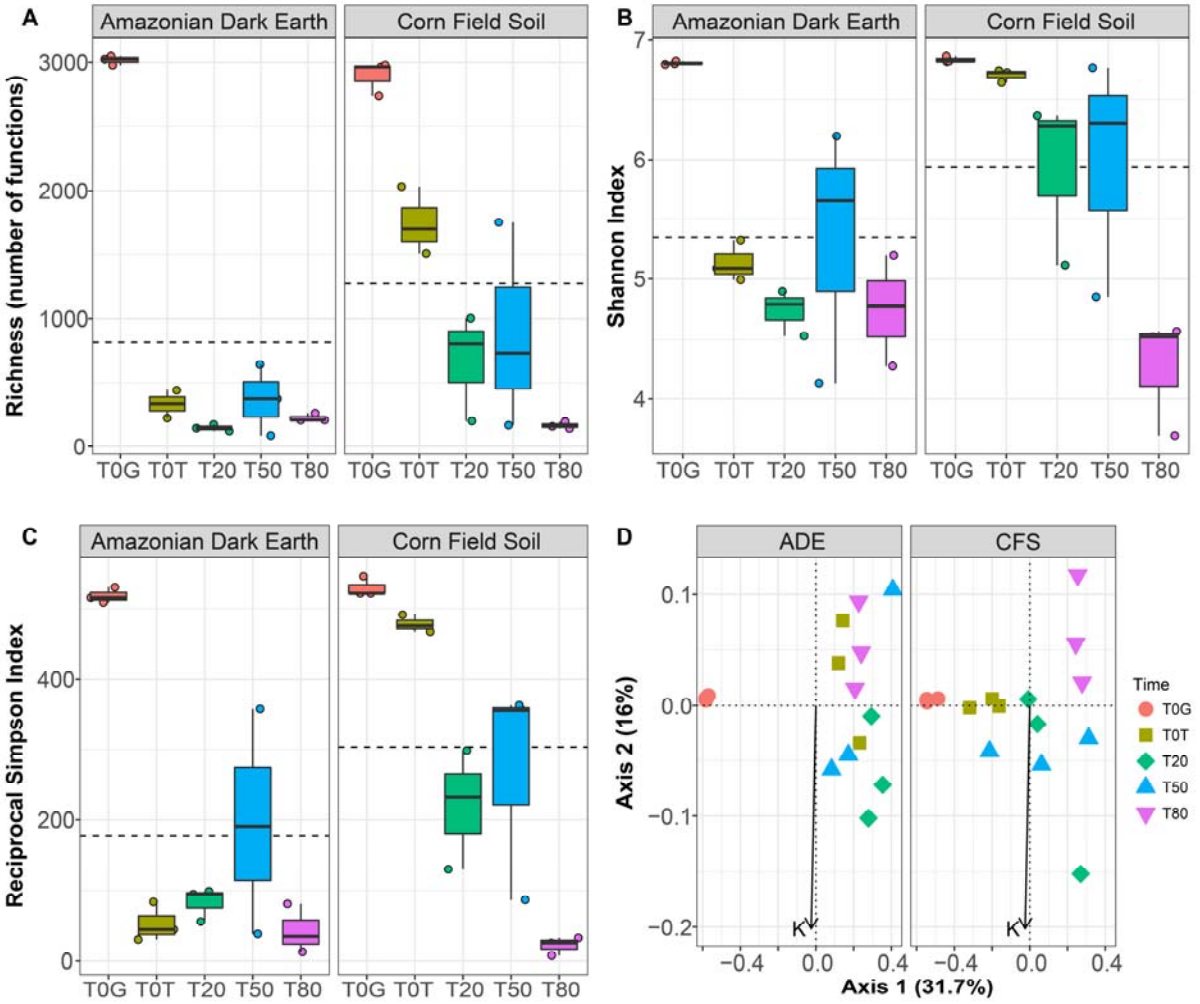
The Amazonia Dark Earth (ADE) and the Corn Field Soil (CFS) were selected due to their major similarities of soil type classification as Latosols (IBGE, 2018) and to their divergent characteristic of soil fertility (Supplementary Table 2). Functionally, there is no difference ($p < 0.05$) on metagenomes dataset between soils (ADE-T0G and CFS-T0T) at Subsystems level 1 (SEED database, neither between CFS initial time of metagenome and metatranscriptome datasets (CFS-T0G and CFS-T0T; Figure 8). But ADE initial time of metatranscriptome dataset (ADE-T0T) increased activities related to RNA metabolism, protein metabolism and respiration from metagenome (ADE-T0G) (Supplementary Figure 3.A) and overexpressed activity of protein metabolism against CFS-T0T (Supplementary Figure 3.B); besides that, no difference was found in other ADE sequential time comparisons (T0T/T20, T20/T50 and T50/T80). On the other hand, activities changes were significative in CFS only at T50/T80 comparison (Supplementary Figure 4), despite the progressive shifts on functional structure overtime.

Figure 8 – Relative abundance profile of sequences matched to Subsystems level 1 (SEED database) from Amazonian Dark Earth (ADE) and Corn Field Soils (CFS). Metagenomic datasets (T0G) from bulk soil and metatranscriptomic dataset (T0T, T20, T50 and T80 correspond to sampling times of 0, 20, 50 and 80 days, respectively) from degraded maize stover



Advancing the profile level 1 to functions, metagenomes (T0G) presented approximately of 3000 functions in both soils. ADE has stronger selectiveness during all degradation process through sampling times, recruiting less than 500 functions in comparison to CFS that starts with 1746±267 functions at CFS-T0T, followed by a reduction at CFS-T20 (669±419), then an increment on variation at CFS-T50 (884±803) – also observed on ADE-T50 (367±278) – and finally a comparable reduction of functions at CFS-T80 (165±27) to ADE-T80 (225±31) (Figure 9.A). Shannon and Reciprocal Simpson indexes corroborate to these results, also reducing and oscillating diversity values overtime (Figure 9.B and C).

Figure 9 – Alpha indexes of (**A**) Richness**,** (**B**) Shannon index, and (**C**) Reciprocal Simpson index; and (**D**) Non-metric multidimensional distance scaling (NMDS) analysis of community composition with physicochemical influence among sampling times through subsystems functions (SEED database) from Amazonian Dark Earth (ADE) and Corn Field Soils (CFS). Metagenomic datasets (T0G) from bulk soil and metatranscriptomic dataset (T0T, T20, T50 and T80 correspond to sampling times of 0, 20, 50 and 80 days, respectively) from degraded straw. See more details about physicochemical characterization in Supplementary Table 2

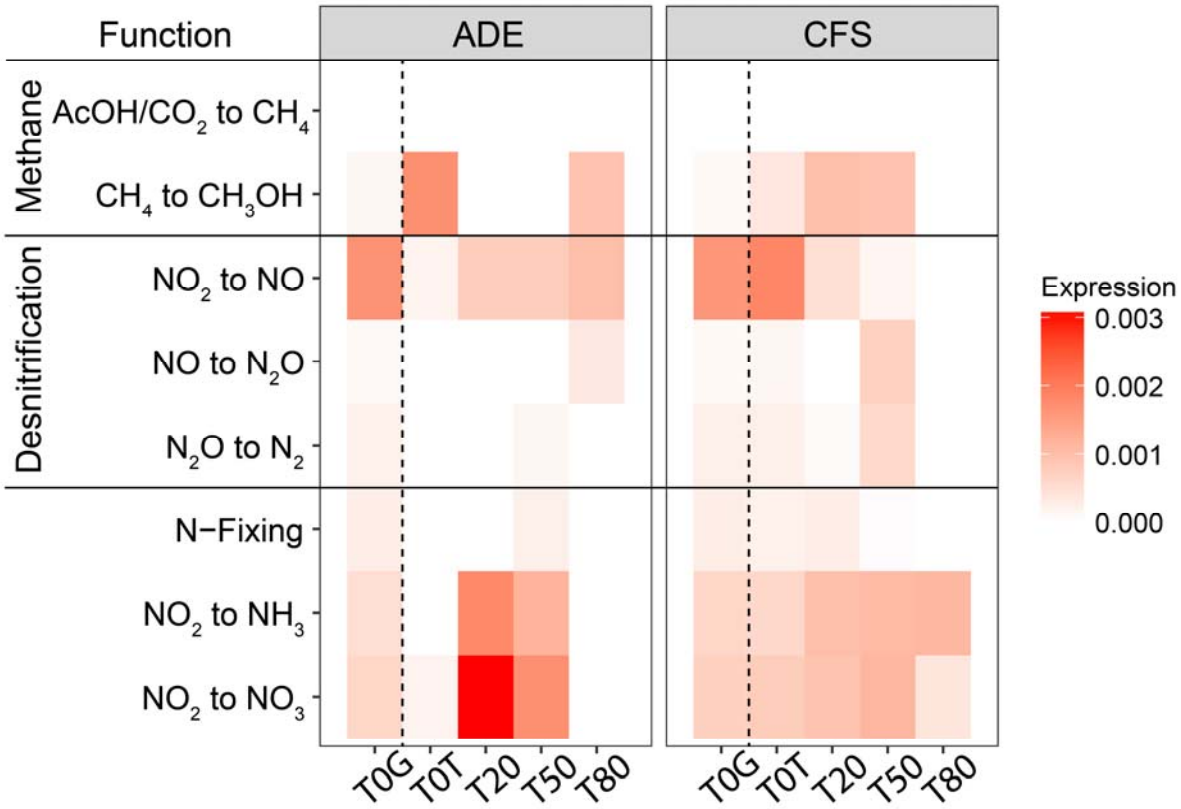


Observing subsystem functions matrices of similarities (Bray-Curtis - $R = 0.328$, $P < 0.001$; Figure 9.D), there is a distinctly separation of metagenome samples for both soils and slightly distinct groups for most of metatranscriptome samples under significative constrained ordination influence of potassium (K).

In order to evaluate decomposition of maize stover and relate to methane (CH_4) and nitrous oxide (N_2O), enzymes expression was retrieved from meta-omics datasets based on methanogenesis ($AcOH/CO_2$ to CH_4), methane monooxygenase (CH_4 to CH_3OH), nitric oxide synthase and denitrification (NO_2^- to NO), denitrification (NO to N_2O), nitrogen

fixation, nitrate and nitrite ammonification (NO_2^- to NH_3), dissimilatory nitrite reductase (NO_2^- to NO_3^-). In general, ADE presented faster response and more intense peaks of enzymes expression than CFS that occurred dispersed overtime. Enzymes involved in methane production has insignificant expression for both soils, meanwhile methane consumption reached peak expression at ADE-T0T and CFS-T20. For nitrous oxide fluxes, the consumption remains dominant unless when production intensifies at later times ADE-T80 and CFS-T50 exceeding consumption expression. Also, transformations of nitrite are very remarkable for ADE-T20, while it is spread overtime for CFS (Figure 10).

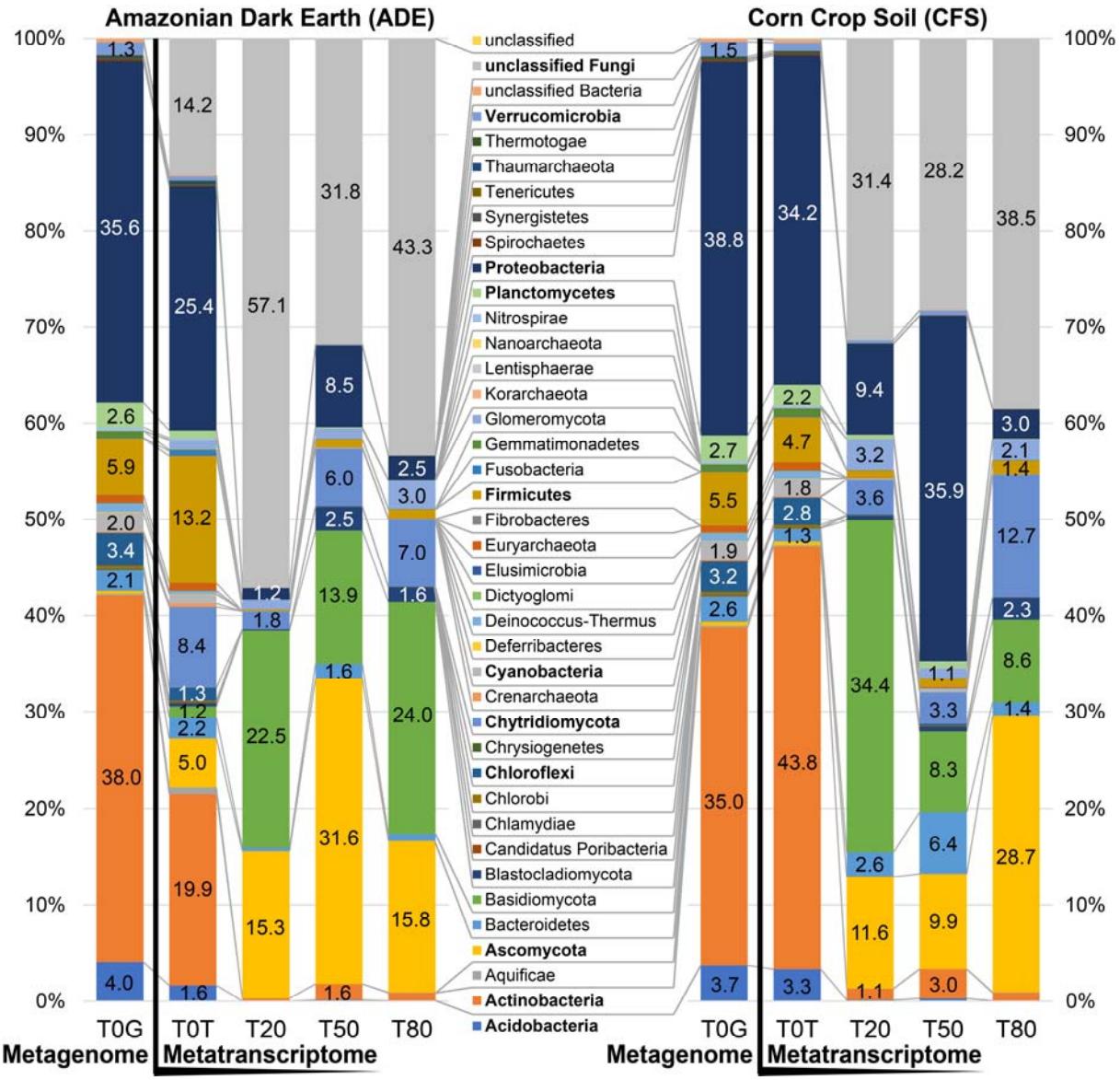
Figure 10 – Heatmap of temporal enzymes relative expression related to microbial Nitrogen and methane (CH_4) cycles conversions on Amazonian Dark Earth (ADE) and Crop Field Soil (CFS). Sequences were matched to subsystem functions (SEED database). Metagenomic datasets (T0G) from bulk soil and metatranscriptomic dataset (T0T, T20, T50 and T80 correspond to sampling times of 0, 20, 50 and 80 days, respectively) from degraded straw



3.3.2 Taxonomic approach

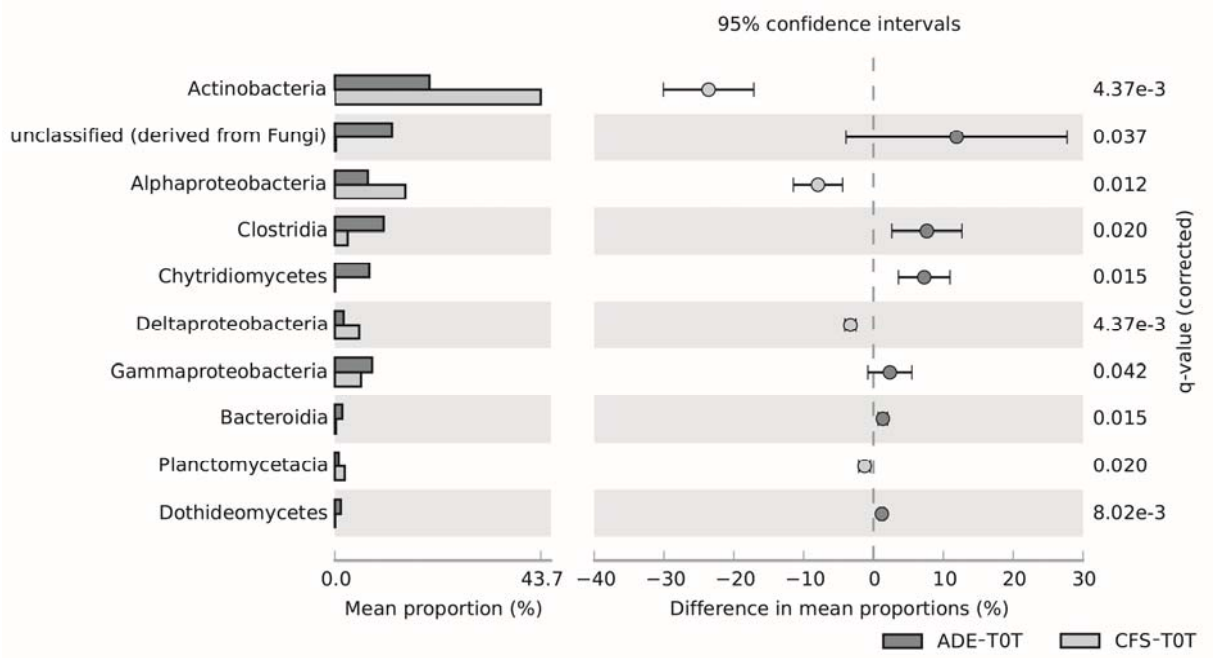
Observing the taxonomic profile based on RefSeq database (Figure 11), metagenomes dataset (T0G), likewise functional profile, presented no significantly difference ($p < 0.05$) between soils having *Actinobacteria* and *Proteobacteria* as the most abundant phyla. In contrast, Fungi become dominant after plant residue addition (T20, T50 and T80) with relative representativeness fluctuation among *Ascomycota*, *Basidiomycota* and unclassified from Fungi phyla.

Figure 11 – Relative abundance of taxonomic affiliation at Phylum level (RefSeq database) from Amazonian Dark Earth (ADE) and Corn Field Soils (CFS). Metagenomic datasets (T0G) from bulk soil and metatranscriptomic dataset (T0T, T20, T50 and T80 correspond to sampling times of 0, 20, 50 and 80 days, respectively) from degraded maize stover



Comparisons between metatranscriptome initial time (T0T), classes overexpressed in ADE-T0T ($q < 0.05$) were unclassified Fungi (12.1%), Clostridia (10.4%), Chytridiomycetes (7.3%), Gammaproteobacteria (7.9%), Bacteroidia (1.6%) and Dothideomycetes (1.3%). While Actinobacteria (43.7%), Alphaproteobacteria (15%), Deltaproteobacteria (5.2%) and Planctomycetacia (2.1%) were overexpressed in CFS-T0T (Figure 12).

Figure 12 – Statistical difference at taxonomic Class level (RefSeq database) between the initial time of metatranscriptome (T0T) from degraded straw under Amazonian Dark Earth (ADE) and Corn Field Soil (CFS) influence. Error bars are standard deviation. q-values were calculated using Storey FDR approach ($p < 0.05$). with effect size filter in difference between proportions (DP < 1.00)



In spite of average variations among time of microbial phyla profile, only paired comparisons between T0G/T0T (Figure 13.A and 14.A) and T0T/T20 (Figure 13.B and 14.B) generated significant differences for both soils. Classes of Actinobacteria, Alphaproteobacteria, Deltaproteobacteria, Planctomycetacia, Chloroflexi and Solibacteres were present in metagenome of ADE soils but unclassified Fungi, Clostridia, Chytridiomycetes, Gammaproteobacteria, Sordariomycetes, Bacteroidia and Dothideomycetes substitute their roles into soil functioning at ADE-T0T metatranscriptome (Figure 13.A). In CFS-T0T, classes of Sphingobacteria, Verrucomicrobia, Opitutae, Spartobacteria and unclassified Thaumarcheota have minor roles for soil niches in opposition to the metagenome CFS-T0G (Figure 14.B)

Figure 13 – Statistical difference at taxonomic Class level (RefSeq database) between (A) metagenome (T0G) and metatranscriptome (T0T), and (B) metatranscriptome of T0T and T20 from degraded straw under Amazonian Dark Earth (ADE) influence. Error bars are standard deviation. q-values were calculated using Storey FDR approach ($p < 0.05$) with effect size filter in difference between proportions (DP < 1.00)

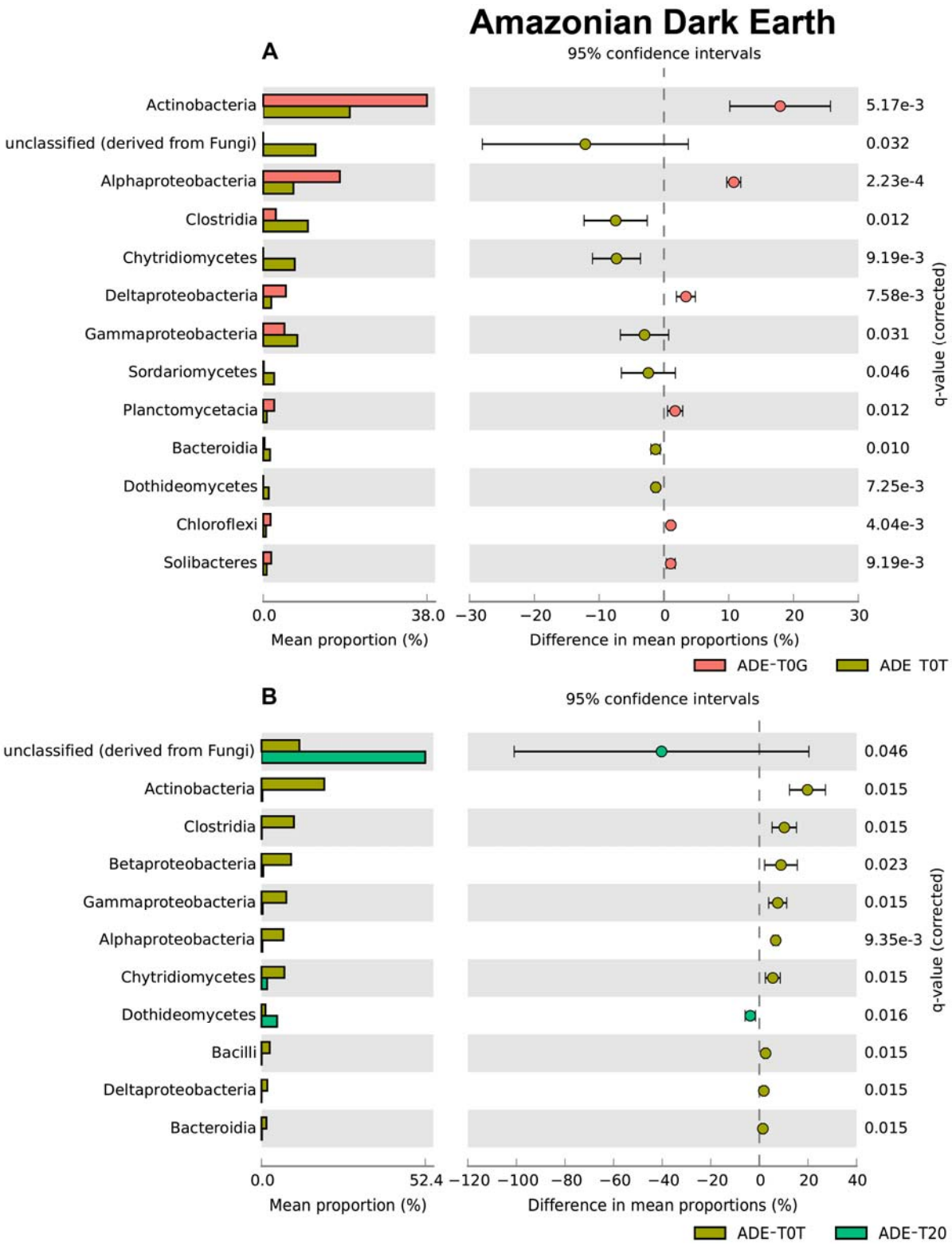
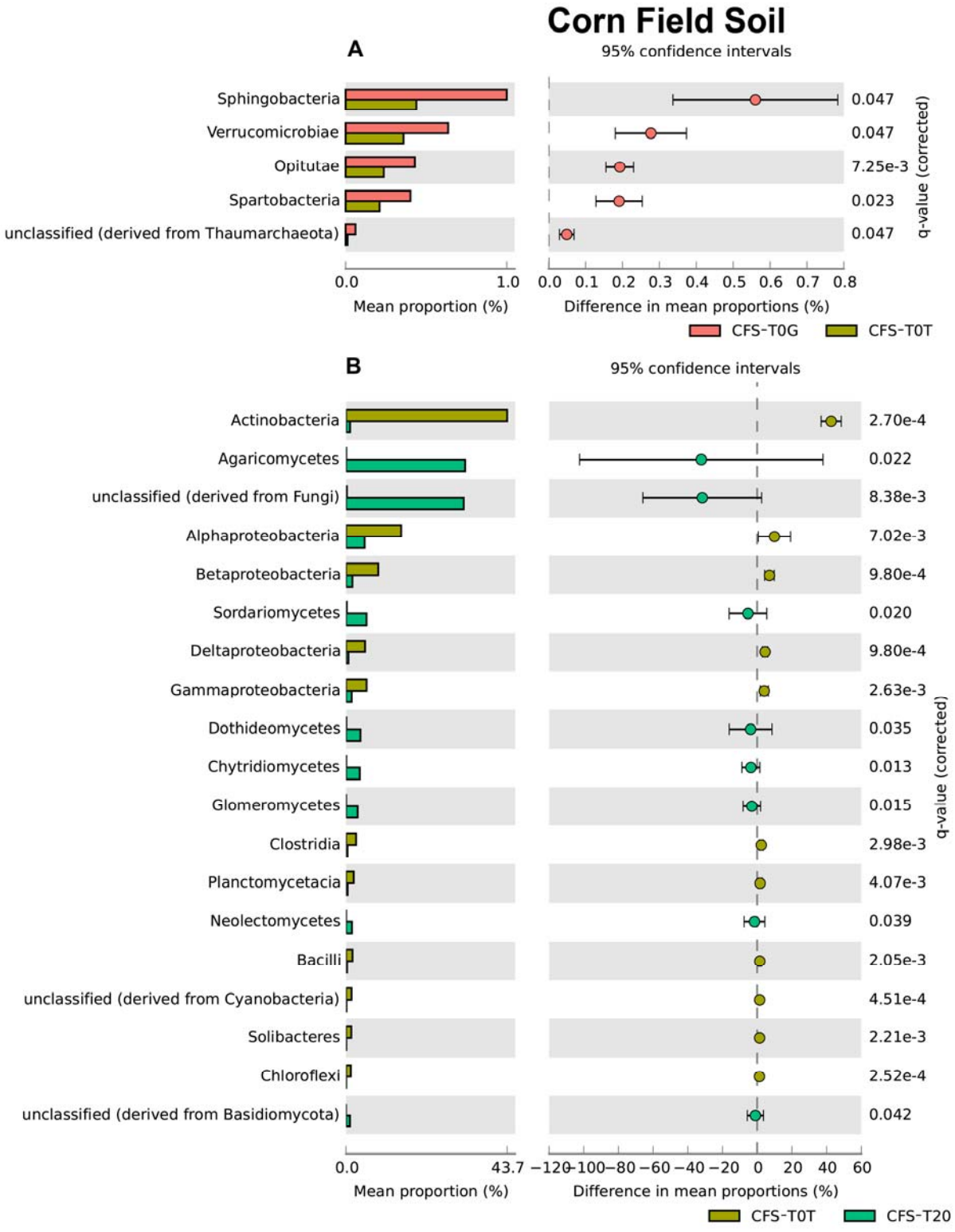


Figure 14 – Statistical difference at taxonomic Class level (RefSeq database) between (A) metagenome (T0G) and metatranscriptome (T0T), and (B) metatranscriptome of T0T and T20 from degraded straw under Corn Fiel Soil (CFS) influence. Error bars are standard deviation. q-values were calculated using Storey FDR approach ($p < 0.05$) with effect size filter in difference between proportions (DP < 1.00)



Focused on cellulose degradation process, unclassified Fungi and Dothiodeomycetes are responsible for major presence (57.4%; Figure 13.B) in the process in ADE and Agaricomycetes, unclassified Fungi, Sordatiomycetes, Dothideomycetes, Chytridiomycetes, Glomeromycetes, Neolectomycetes and unclassified Basidiomycota would play important roles in CFS (83.3%; Figure 14.B).

3.4 Discussion

Biodegradation process of complex cellulose substrates recruit microbial communities accordingly to substrate resilient aspect as initial degraders for cellulose and hemicellulose and later degraders for resilient lignin (VAN DER HEIJDEN et al., 2008). Not surprisingly, RNA and protein metabolism are promptly recruited as opening activities from metatranscriptome datasets for both soils. But, our functional profile pointed ADE as faster responsive soil overtime than CFS, reducing the number and diversity of functions as a specialized machinery for plant residue degradation process while CFS seems to proceed a gradually selection of functions during all evaluation period of the experiment.

Our focus on methane and nitrogen cycle expression analysis resulted as hypothesized in previous assumption (Study I) that plant residue of maize could improve crop yield with neutral emission of CH_4 and N_2O, likely to biochar (also aromatic compound carbon-rich solid that is produced by pyrolysis biomass) as soil amendment for reducing N_2O emissions (HARTER et al., 2017). However, the nitrogen fixing bacteria were not strongly related to denitrifying fuc and their presence is low during plant residue decomposition as well. Moreover, nitrate and nitrite ammonification, and nitrite reductase are in alternative pathways, obtaining ammonia (NH_3) and nitrate (NO_3^-) more expressed (2 or 3-fold) than denitrification process for NO^- to N_2O transformation, indicates an avoidance of N_2O emissions during degrading process. Additionally, the gradual expression of nitrate and nitrite ammonification and nitrite reductase overtime indicates a constantly and not gaudy liberation of N-forms for plant assimilation that restrict N availability for N_2O formation pathway. Against it, the initial denitrification process responsible for NO^- synthesis is well expressed, but the denitrification process to N_2O is poorly expressed most of time. This NO^- availability from soil normal functions can act as positive regulator for root hair and development to facilitate nitrogen uptake in plants from (NH_3 and NO_3^-) (LOMBARDO et al., 2006; SUN et al., 2017) that could be responsible for plant growth under straw treatments on Study I, but it requires attention to not become a N_2O source.

As previously reported, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacterioidetes*, *Clostridia*, *Ascomycota* and *Basidiomycota* phyla are the main microbes involved on lignocellulose degradation by acting in pathways as polysaccharide and glycoside degradation, lignin depolymerization, aromatic compounds oxidation (KELNNER et al., 2008; KANOKRATANA et al., 2011; CASTILLO et al., 2013; MA et al., 2013; MARTINS et al., 2013; WU; HE, 2013). Besides that, their presence on samples is variable resulting in few significative differences that would be associated to hotspots creation due to competition for substrate availability and formation of derived substances as products of different microorganism (HIBBING et al., 2010).

3.5 Conclusion

Overall, Amazonian Dark Earth (ADE) and corn field soils (CFS) has similar structure and functional profiles in metagenome approach, although differences overtime in metatranscriptome indicates that, inclusive for functions related to methane and nitrous oxide metabolisms, ADE is faster responsive for maize stover addition than CFS and the degradation process is prevalent performed by Fungi. Apparently, few or none correlation were found between nitrogen fixing and others pathways, but nitrite reductase and nitrate and nitrite ammonification were overwhelming expressed and would avoid nitrogen conversions to N_2O. Therefore, the enzymes involved in cellulose decomposition seem to not be the responsible for expressive increments on N_2O emissions, although we cannot underestimate the existence of denitrification pathway to N_2O formation.

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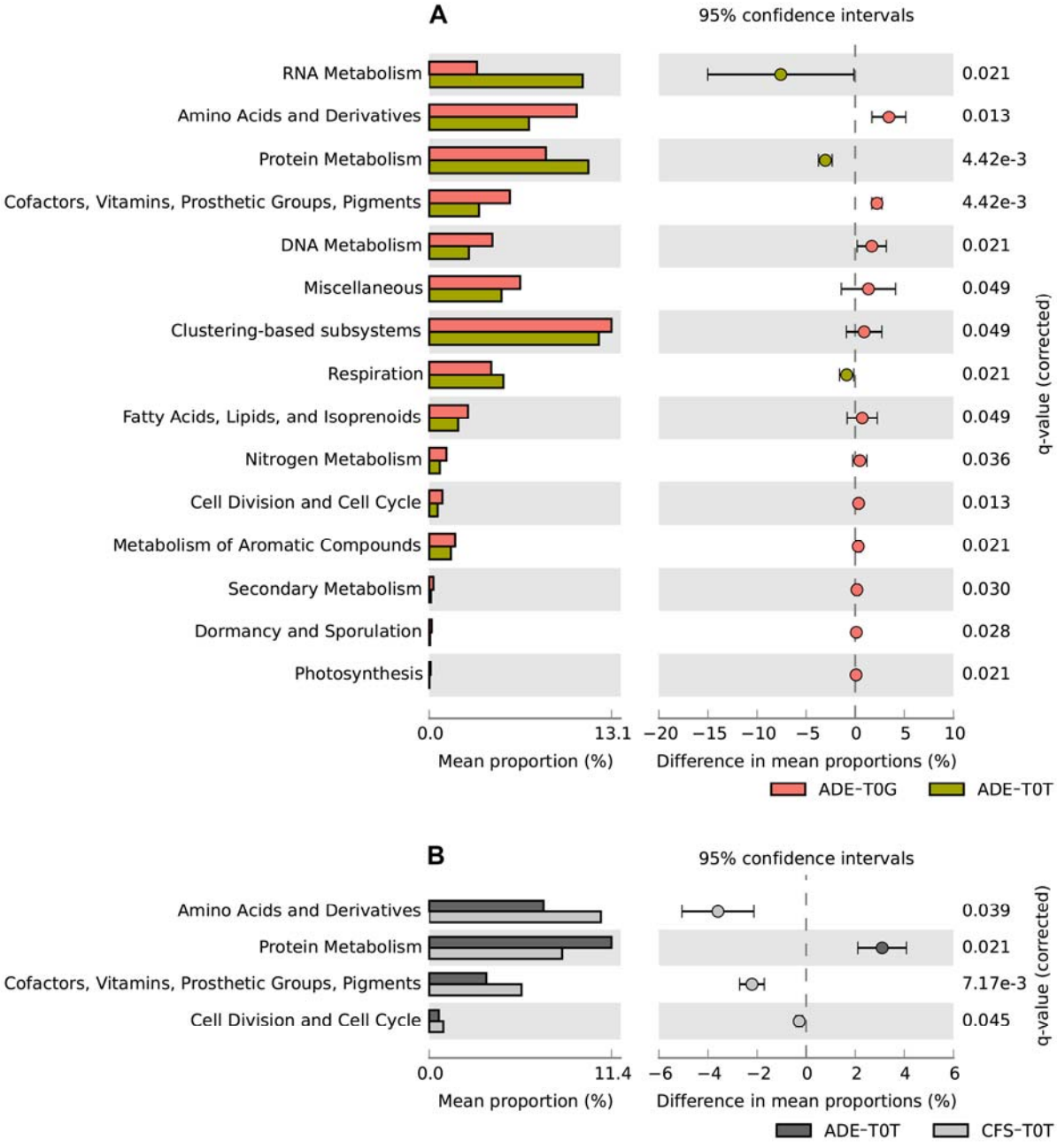
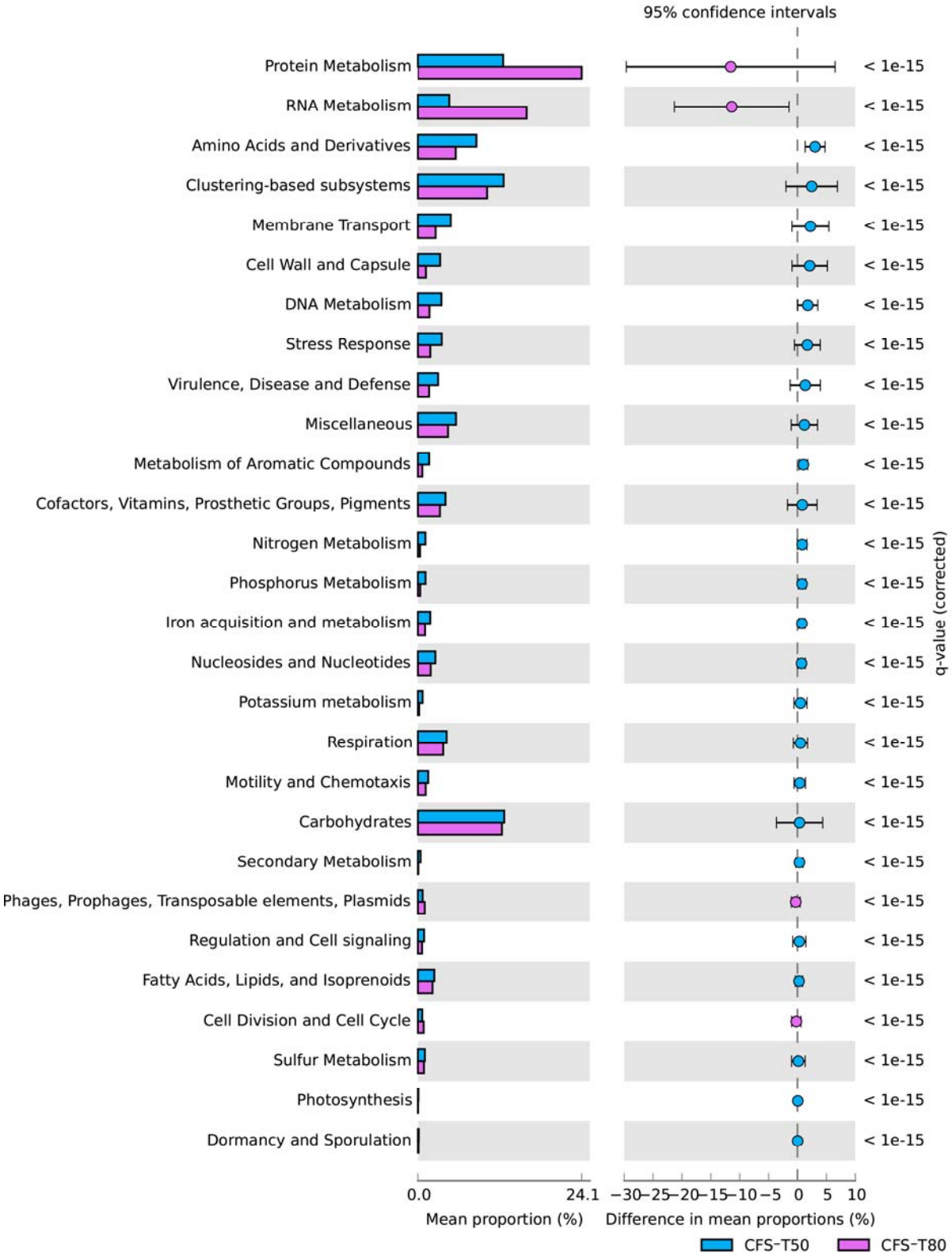
Supplementary Figure 3 – Statistical difference between (A) metagenome (T0G) and metatranscriptome (T0T) from degraded maize stover under influence of Amazonian Dark Earth (ADE), and (B) metatranscriptome from ADE and Corn Field Soil (CFS) of representative groups from Subsystems level 1 (SEED database). Error bars are standard deviation. q-values were calculated using Storey FDR approach ($p < 0.05$) between time and Benjamin-Hochberg for soils comparison.



Supplementary Figure 4 – Statistical difference between metatranscriptome T50 and T80 of representative groups from Subsystems level 1 (SEED database) from degraded maize stover under Corn Field Soil (CFS) influence. Error bars are standard deviation. q-values were calculated using Storey FDR approach ($p < 0.05$)



Supplementary Table 2 – Average soil chemical characteristics of treatments at V5 Stage. Values are mean ±SE, n = 3. Columns mean followed by same letter or letter absence are not significantly different ($p < 0.05$) bases on Tutkey HSD test and Dunn test for normal and non-normal values, respectively. Ca, Mg, Al, potential acidity (H+Al), sum of bases (SB) and cation exchange capacity (CEC) are expressed in $mmolc.kg^{-1}$. P is expressed in $mg.kg^{-1}$. Organic matter (OM) is expressed in $g.kg^{-1}$.

Treatment	pH	CEC	V	M	OM	P	K	Ca	Mg	Al	H+Al	SB
ADE - T0G	5.4±0.1 ab	134.7±1.2 a	74.3±1.2	0.0	39.6±0.4 a	413.3±16.9 a	3.7±0.3	78.7±2.3 a	18.3±0.9 a	<1	34.0±1.2 a	100.7±1.9 a
ADE- T0T	5.4±0.1 ab	133.0±1.5 a	74.0±1.0	0.0	38.3±0.7 a	414.0±7.8 a	3.7±0.9	77.3±2.4 a	18.0±0.6 a	<1	34.0±1.2 a	99.0±2.1 a
ADE - T20	5.3±0.0 ab	134.3±1.9 a	74.0±0.6	0.0	38.8±0.6 a	415.7±11.7 a	3.7±1.5	77.7±1.5 a	19.0±1.0 a	<1	34.0±1.2 a	100.3±0.9 a
ADE - T50	5.2±0.1 b	131.7±2.9 a	73.7±1.3	0.0	38.2±0.4 a	419.0±2.1 a	3.7±0.9	77.0±2.1 a	17.0±1.2 a	<1	34.0±1.2 a	97.7±3.5 a
ADE - T80	5.2±0.1 b	129.7±1.2 a	73.3±0.9	0.0	37.5±0.4 a	411.3±12.9 a	2.0±0.6	75.3±1.5 a	18.3±1.20 a	<1	34.0±1.2 a	95.7±0.3 a
CFS - T0G	6.1±0.1 a	44.0±1.5 b	62.3±1.9	0.0	7.7±0.3 b	44.3±4.5 b	5.0±1.5	16.7±0.7 b	6.0±0.6 b	<1	16.3±1.2 b	27.7±0.9 b
CFS- T0T	6.2±0.0 a	42.3±1.2 b	61.3±1.9	0.0	8.0±0.6 b	43.7±1.7 b	4.3±1.2	16.3±0.7 b	5.3±0.9 b	<1	16.3±1.2 b	26.0±0.0 b
CFS - T20	6.2±0.0 a	43.0±1.7 b	61.7±1.7	0.0	6.7±0.3 b	42.7±2.0 b	4.3±0.7	16.7±0.3 b	5.7±0.3 b	<1	16.3±1.2 b	26.7±0.7 b
CFS - T50	6.1±0.0 a	42.0±1.2 b	60.7±2.7	0.0	7.3±0.3 b	42.0±2.6 b	4.0±1.2	15.7±1.2 b	6.0±1.2 b	<1	16.3±1.2 b	25.7±1.5 b
CFS - T80	6.1±0.1 a	41.0±1.5 b	60.0±1.7	0.0	6.7±0.9 b	40.0±1.0 b	3.3±0.3	15.0±0.6 b	6.3±0.7 b	<1	16.3±1.2 b	24.7±0.7 b

4 PROSPECTING BACTERIA ASSOCIATED TO CELLULOSE DEGRADATION AND N_2O MITIGATON FROM AMAZONIAN DARK EARTH AND BRAZILIAN CORN FIELD SOIL

Abstract

Growing bacteria could be tricky; once the simulation of environmental factors is hard to perform and provide for suitable conditions to microorganisms' growth. Culturable-independent methods can be explored for information about microorganisms' biology and their preferences. In this study were used the addition of micronutrients (Fe, Cu, Zn and Mo) in culture media to supplement microorganisms' synthesis of enzymes related denitrifying and nitrogen fixation metabolisms under low oxygen conditions to reduce aerobic bacteria growth that are commonly cultured that would be used in future studies. As results, in total 420 microorganisms were isolated from Amazonian Dark Earth (ADE) and corn field soil (CFS) using modified NFCC and CMC media. From this, 62 strains (96 selected to 16S rRNA gene sequencing) of isolated strains were identified belonging to genera *Bacillus*, *Paenibacillus*, *Lysinibacillus*, *Streptomyces*, *Arthrobacter*, *Luteibacter*, *Pseudomonas* and *Mycobacterium*, however complete sequencing must be performed to confirm their species. The gene detection by conventional PCR reveals the success of isolation for denitrifying bacteria with *nosZ* gene. Other genes would be tested for functional characterization and correlate to their potential for GHGs fluxes to eventually be combined for inoculation products development.

Keywords: Modified culture media. Hypoxic condition. Nitrogen cycle. Cellulose degradation

4.1 Introduction

Starting from the example of microbial inoculant as plant growth-promoting rhizobacteria (PGPR) that live closely to rhizosphere of plants due to roots exudation to select beneficial microorganisms to plant development; in the same way, degradation process also acts selectively for specialized microorganisms that are capable to cleave molecular bonds of lignocellulose through enzymatic activity. Biodegradation process of complex lignocellulose substrates recruit microbial communities accordingly to substrate resilient aspect as initial degraders for cellulose and hemicellulose and later degraders for resilient lignin (VAN DER HEIJDEN et al., 2008). Lignocellulolytic microorganisms can synthesize several distinct enzymes which cooperate to plant residue degradation acting in cellulose, hemicellulose and lignin.

Gathering all the information from previous Studies I and II, the prospection for microorganisms that are capable to interact in plant development, not necessarily a synergic effect but several microorganisms are capable to provide plant growth substances and respond to plant residue presence; the sought for beneficial functions in joker microorganisms or multi-composition should be the focus to improve crop yield or at least the crop quality (RODRÍGUEZ CÁCERES et al, 1996; TRABELSI and MHAMDI, 2013; DARTORA et al., 2016).

Difficulties found on growing bacteria methods are very common because there is a great chance of failure in attempts to mimic environmental factors with affordable conditions for their survival (VARTOUKIAN et al., 2010). In this way, several modifications on methods (i.e. media composition, oxygen availability, synergic microbial interactions) are being studied to access these uncultured bacteria to provide microorganisms growth (STEWART, 2012; MORI and KAMAGATA, 2014). By the way, positive results from metagenome and metatranscriptome prospection information from previous studies and the need for greenhouse gases (GHGs) consumers directed the search for methodologies in microbial isolation.

In this study, we used the addition of micronutrients like iron (Fe), copper (Cu), zinc (Zn) and molybdenum (Mo) for specific enzymes as previously described (GLASS; ORPHAN, 2012) to retrieve specific bacteria involved in nitrous oxide metabolism in specific media for cellulolytic and nitrogen related bacteria under hypoxic conditions; as result of previous Studies I and II.

4.2 Materials and Methods

4.2.1 Sampling

Soil samples were collected from adjacent soils of litter bags containing degraded maize stover (Straw-degradation treatment) or not (Control group). From a maize stover decomposition experiment incubated for 80 days at 25ºC and humidity controlled to 80% of field capacity into two different soil types: bulk-rhizosphere top-soil (25 cm) of corn field (CFS) (22º50'28.22"S, 48º1'1.7"W) from Anhumas Experimental Station of the Department of Genetics of Luiz de Queiroz College of Agriculture (ESALQ) – University of São Paulo (USP) – this type soil is classified as Dystrophic Red-Yellow Latosol. Dystrophic Latosols represents the major part of soils used for corn production in Brazil (IBGE, 2018; CONAB, 2018); and bulk top-soil of Amazonian Dark Earth (ADE) from Hatahara site (03º16.494'S60º12.340'W) – an anthropic soil derived from a Dystrophic Yellow Latosol (IBGE, 2018). The soils were maintained at 4ºC and transported to Soil EcoGenomics Laboratory at UCDavs, Davis, CA, USA for microbial isolation procedures.

4.2.2 Isolation of microorganisms under hypoxic condition

Soil (10 g) was added to 100 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7), vigorously mixed for 10 min, and diluted in PBS. Serial dilutions from 10^{-3} to 10^{-7} were used for all subsequent procedures. Trace Metal Solution were prepared to compose all media (2.76 g Fe-EDTA, 190 mg $CoCl_2.6H_2O$, 100 mg $MnCl_2.4H_2O$, 148 mg $ZnSO_4.7H_2O$, 36 mg $Na_2MoO_4.2H_2O$, 24 mg $NiCl_2.6H_2O$, 2 mg $CuCl_2.2H_2O$, 6 mg, H_3BO_3, 10 mL HCl 25%, 1 L deionized water). 0.5% Alcoholic Bromothymol Blue Solution (0.5 g Bromothymol Blue, 50 mL EtOH 95%, 0.4 g KOH, 50 mL distilled water) were used to pH control of NFCC media and 0.1% Congo Red Solution (0.1 g Congo Red, 99.9 mL distilled water) were used to AC/CMC media.

Solid Nitrogen-free combined carbon (NFCC) medium modified from Mirza and Rodrigues (2012) (5 g Malic Acid, 2.5 g Glucose, 4.7 g KOH, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g, NaCl, 0.02 g CaCl, 1 mL Trace Metal Solution, 2 mL 0.5% Alcoholic Bromothymol Blue Solution, 5 g Gellan Gum, 1 L distilled water). Cellulose degraders were isolated using Avicel (AC) and Carboxymethyl Cellulose (CMC) medium modified from Ventorino et al. (2015) (5 g CMC or 5 g Avicel (microcrystalline cellulose), 1 g $NH_4H_2PO_4$,

0.2 g KCl, 1 g $MgSO_4.7H_2O$, 1 g Yeast Extract, 1 mL Trace Metal Solution, 1 mL 0.1% Congo Red Solution, 6 g Gellan Gum, 1 L distilled water).

Solid media were prepared on petri dishes (90 mm x 15 mm) then were inoculated with 100 µL of soil serial dilutions and incubated under hypoxic conditions (2% O_2 and 98% N_2) in a vinyl hypoxic chamber fitted with an automated oxygen sensor and controller (Coy Laboratory Products, Grass Lake, MI) for 21 days to NFCC media and 7 days to AC/CMC. Isolated colonies were transferred successively to other plates until certify their purity.

4.2.3 Cell culture and glycerol frozen stock

Each isolated culture was grown in triplicate using microtubes containing liquid version of the same initial isolation media. From this, one microtube was maintained at 4ºC *in vivo* until viability check after thaw of -80ºC frozen stock. While, two microtubes containing 1 mL of culture suspensions were centrifuged at 4.000 rpm for 5 min and discard the supernatant. Then, one microtube was resuspended in liquid media with 20% glycerol and transferred to two cryovials with 0.5 mL each of culture suspension for frozen stock at -80ºC. And the other centrifuged microtube was -20ºC stored until DNA extraction for molecular analysis.

4.2.4 DNA extraction

The isolates were plated to be grouped by similar visual phenotypic characteristics. Two milliliters of the liquid cell culture from each unique isolate was harvested by centrifugation at 4,000 rpm for 10 min and performed the DNA extraction following gram-positive bacteria of QIAamp DNA Mini Kit (QIAgen) with final elution in 150 µL. Aliquots were sent to Brazil to perform 16S sequencing for identification of the isolates.

4.2.5 PCR screening of functional genes

PCR of functional genes were applied to detect their presence in isolated microorganisms' genomes. The functional genes: *nifH* for nitrogen fixation, *nosZ* for denitrifying bacteria, *GH10* and *GH11* for glycoside hydrolases family 10 and 11 whose enzymes are responsible for endo-β-1,3-xylanases and endo-β-1,4-xylanases activities (and

were observed in metatranscriptome of maize degradation), respectively (Table 2). The PCR reactions using 1x Reaction Buffer, 200 µM dNTPs, 0.5 µM forward and reverse primer, 1 U Taq DNA Polymerase (GenScript), 1 µL template DNA, completed with deionized water to 20 µL final reaction volume. The thermocycler conditions: for *nifH* were 5 min of denaturation at 95ºC, followed by 35 rounds of temperature cycling (95°C for 15 s, 62°C for 25 s, and 72°C for 45 s) and a final extension at 72°C for 7 min; for *nosZ* were 5 min of denaturation at 95ºC, followed by 35 rounds of temperature cycling (95°C for 45 s, 62°C for 60 s, and 72°C for 30 s) and a final extension at 72°C for 7 min, for *GH10* and *GH11* were 4 min of denaturation at 95ºC, followed by 12 rounds of temperature cycling (95ºC for 30 s, 56ºC (58ºC to *GH11*) for 30 s, and 72ºC for 30 s) decreasing -0.5ºC each cycle then 28 rounds of temperature cycling (95ºC for 30 s, 50ºC (52ºC to *GH11*) for 30 s, and 72ºC for 30 s) and a final extension at 72°C for 6 min. Aliquots (5 µl) of PCR products were checked on GelRed-stained 1% agarose gels.

Table 2 – Sequence of primers for functional genes detection by PCR

Gene	Primer	Sequence	Reference
nifH	PolF	5' TGCGAYCCSAARGCBGACTC 3'	POLY et al., 2001
	PolR	5' ATSGCCATCATYTCRCCGGA 3'	
nosZ	nosZ2F	5' CGCRACGGCAASAAGGTSMSSGT 3'	HENRY et al., 2006
	nosZ2R	5' CAKRTGCAKSGCRTGGCAGAA 3'	
GH10	X10-F	5' CTACGACTGGGAYGTNIBSAAYGA 3'	WANG et al., 2010
	X10-R	5' GTGACTCTGGAWRCCIABNCCRT 3'	
GH11	X11-F	5' AACTGCTACCTGKCNITNTAYGGNTGG 3'	
	X11-R	5' CCGCACGGACCAGTAYTGNKIRAANGT 3'	

4.2.6 Amplification of 16S rRNA gene and sequencing

The 16S rRNA gene of bacterial isolates was amplified using primer set: FD1 (5' AGAGTTTGATCCTGGCTCAG 3') and RD1 (5' AAGGAGGTGATCCAGCC 3') for bacterial identification by sequencing. The PCR reactions were carried out using 1x Reaction Buffer, 1.5 mM $MgCl_2$ 200 µM dNTPs, 0.2 µM forward and reverse primer, 1 U Platinum Taq DNA Polymerase (Invitrogen, Vilnius, LT), 1 µL template DNA, completed with deionized water to 35 µL final reaction volume. The thermocycler conditions were 5 min of denaturation at 94ºC, followed by 30 rounds of temperature cycling (94°C for 30 s, 55°C for 45 s, and 72°C for 60 s) with final extension of 10 min. Sequencing reactions were carried out using BigDye

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following manufacturer's protocol. And subsequently sequenced using FD1 primer and performed in 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

4.2.7 Identification of strains

Sequences of 16S rRNA gene were treated using Phred, Phrap, and Consed (EWING et al., 1998; EWING; GREEN, 1998; GORDON et al., 1998). And aligned to the Ribosomal Database Project (RDP – http://rdp.cme.msu.edu/) and Blastn (https://blast.ncbi.nlm.nih.gov/)

4.3 Results

4.3.1 Isolation of microorganisms and selection of unique phenotypes

In total, 420 pure isolates were obtained using modified media: 80 from NFCC medium, 104 from AC medium, and 236 from CMC medium (Table 3). The isolates grouped by similar visual phenotypic characteristics, totalizing in 122 unique phenotypes.

Table 3 – Number of isolates obtained per soil type and media under hypoxic conditions (2% O_2). CFS – Corn Field Soil, ADE – Amazonian Dark Earth

Media	CFS	ADE
NFCC	66	14
AC	68	36
CMC	119	117

4.3.2 Detection of functional genes and strains identification

Results of functional genes detection from the 122 isolates are displayed in Tables 4, 5 and 6 with identification of partial 16S rRNA gene of 96 isolates in Table 7.

Table 4 – Genes detected in strains with different phenotypic features isolated from Nitrogen-Free Combined Carbon (NFCC) modified from Mirza and Rodrigues (2012) during 21 days of initial incubation

Strain	Soil	Type	*nifH*	*nosZ*	*GH10*	*GH11*
N02	CFS	Control		✓		
N03	CFS	Control		✓		
N06	CFS	Control		✓		
N09	CFS	Control		✓	✓	
N12	CFS	Control				
N13	CFS	Control				
N14	CFS	Control		✓		
N17	CFS	Control		✓		
N18	CFS	Control		✓		
N19	CFS	Control		✓		
N20	CFS	Control		✓		
N22	CFS	Control				
N26	CFS	Control		✓		
N27	CFS	Control		✓		
N28	CFS	Straw		✓		✓
N29	CFS	Straw		✓		
N31	CFS	Straw		✓		
N34	CFS	Straw		✓		
N38	CFS	Straw		✓	✓	
N39	CFS	Straw		✓		
N40	CFS	Straw		✓		
N41	CFS	Straw		✓		
N42	CFS	Straw				
N45	CFS	Straw		✓		
N47	CFS	Straw		✓		
N48	CFS	Straw		✓	✓	✓
N56	CFS	Straw		✓		
N60	CFS	Straw		✓		✓
N61	CFS	Straw		✓		
N63	CFS	Straw				
N64	CFS	Straw		✓		
N65	CFS	Straw	✓	✓	✓	✓
N66	CFS	Straw				
N67	ADE	Control				
N68	ADE	Control		✓		
N75	ADE	Straw		✓		
N80	ADE	Straw		✓		

Table 5 – Genes detected in strains with different phenotypic features isolated from Avicel (AC) modified from Ventorino et al (2015) during 7 days of initial incubation

Strain	Soil	Type	*nifH*	*nosZ*	*GH10*	*GH11*
A007	CFS	Control		✓		
A008	CFS	Control		✓		
A012	CFS	Control		✓		
A015	CFS	Control		✓		
A017	CFS	Control	✓	✓		
A019	CFS	Control		✓		
A020	CFS	Control		✓		
A031	CFS	Control		✓		
A032	CFS	Control		✓		
A034	CFS	Control		✓		
A035	CFS	Control		✓		
A036	CFS	Control		✓		
A36B	CFS	Control		✓		
A041	CFS	Straw		✓		
A042	CFS	Straw		✓		
A043	CFS	Straw		✓		
A045	CFS	Straw		✓		
A046	CFS	Straw	✓	✓		
A049	CFS	Straw		✓		
A054	CFS	Straw		✓		
A058	CFS	Straw		✓		
A061	CFS	Straw		✓		
A068	CFS	Straw		✓		
A077	ADE	Control		✓		
A084	ADE	Straw		✓		
A091	ADE	Straw		✓		✓
A092	ADE	Straw		✓		
A094	ADE	Straw		✓		
A098	ADE	Straw	✓	✓		
A099	ADE	Straw				
A101	ADE	Straw	✓	✓		

Table 6 – Genes detected in strains with different phenotypic features isolated from Carboxymethyl Cellulose (CMC) modified from Ventorino et al. (2015) during 7 days of initial incubation

Strain	Soil	Type	*nifH*	*nosZ*	*GH10*	*GH11*	Strain	Soil	Type	*nifH*	*nosZ*	*GH10*	*GH11*
C002	CFS	Control	✓	✓			C129	ADE	Control		✓		
C003	CFS	Control		✓			C131	ADE	Control		✓		
C008	CFS	Control		✓	✓		C136	ADE	Control		✓		
C011	CFS	Control		✓			C143	ADE	Control		✓		
C014	CFS	Control	✓	✓			C153	ADE	Control	✓	✓		
C015	CFS	Control	✓	✓			C154	ADE	Control		✓		
C016	CFS	Control	✓	✓			C155	ADE	Control		✓		
C020	CFS	Control		✓			C158	ADE	Control		✓		
C024	CFS	Control	✓	✓			C161	ADE	Control		✓		
C031	CFS	Control		✓			C169	ADE	Control		✓		
C032	CFS	Control		✓	✓	✓	C177	ADE	Straw		✓		
C033	CFS	Control			✓		C180	ADE	Straw	✓	✓		
C054	CFS	Control		✓			C185	ADE	Straw		✓		
C058	CFS	Control		✓	✓		C188	ADE	Straw		✓		
C059	CFS	Control	✓	✓			C195	ADE	Straw		✓		
C060	CFS	Control		✓			C201	ADE	Straw				
C066	CFS	Straw	✓	✓			C203	ADE	Straw		✓		
C080	CFS	Straw		✓			C210	ADE	Straw		✓		
C082	CFS	Straw		✓			C213	ADE	Straw	✓	✓		
C083	CFS	Straw		✓			C215	ADE	Straw	✓	✓		
C084	CFS	Straw		✓			C217	ADE	Straw		✓		
C090	CFS	Straw		✓			C222	ADE	Straw		✓		
C091	CFS	Straw					C226	ADE	Straw		✓		
C101	CFS	Straw		✓			C233	ADE	Straw		✓		
C109	CFS	Straw		✓			C234	ADE	Straw		✓		
C116	CFS	Straw		✓		✓	C235	ADE	Straw		✓		
C120	ADE	Control		✓			C236	ADE	Straw		✓		

Table 7 – Identification of 96 strains obtained from isolation media

Strain	Type	Specie	Identity
N02	Partial	*Bacillus pumilus*	99%
N12	Partial	unidentified	-
N14	Partial	*Bacillus cereus*	98%
N17	Partial	*Bacillus circulans*	88%
N18	Partial	*Bacillus safensis*	98%
N19	Partial	*Bacillus subtilis*	89%
N20	Partial	*Bacillus safensis*	89%
N26	Partial	*Bacillus pumilus*	95%
N27	Partial	*Bacillus cereus*	89%
N34	Partial	*Bacillus sp.*	96%
N39	Partial	unidentified	-
N40	Partial	*Bacillus sp*	90%
N41	Partial	*Bacillus cereus*	99%
N45	Partial	*Bacillus velezensis*	97%
N47	Partial	*Streptomyces bungoensiis*	99%
N60	Partial	*Bacillus amyloliquefaciens*	97%
N63	Partial	*Luteibacter rhizovicinus*	99%
N64	Partial	*Bacillus amyloliquefaciens*	98%
N65	Partial	*Paenibacillus alvei*	97%
N66	Partial	*Bacillus cereus*	99%
N67	Partial	*Paenibacillus lautus*	95%
N75	Partial	*Bacillus thurigiensis*	99%
N80	Partial	*Lysinibacillus fusiformis*	98%
A007	Partial	*Bacillus thurigiensis*	99%
A015	Partial	*Bacillus subtilis*	90%
A017	Partial	unidentified	-
A019	Partial	*Paenibacillus xylanilyticus*	93%
A020	Partial	unidentified	-
A031	Partial	*Bacillus methylotrophicus*	96%
A034	Partial	unidentified	-
A036	Partial	*Bacillus pumilus*	96%
A041	Partial	unidentified	-
A042	Partial	*Bacillus cereus*	98%
A043	Partial	unidentified	-
A045	Partial	*Lysinibacillus fusiformis*	96%
A046	Partial	unidentified	-
A054	Partial	*Streptomyces sp.*	91%
A058	Partial	unidentified	-
A061	Partial	*Bacillus anthracis*	97%
A077	Partial	unidentified	-
A084	Partial	*Bacillus subtilis*	93%
A092	Partial	unidentified	-
A094	Partial	*Paenibacillus polimixa*	88%
A098	Partial	unidentified	-
A099	Partial	*Bacillus thurigiensis*	99%
A101	Partial	unidentified	-

continue

C002	Partial	unidentified	-
C003	Partial	unidentified	-
C008	Partial	*Mycobacterium anyagense*	96%
C011	Partial	*Arthrobacter pokkalii*	98%
C014	Partial	*Arthrobacter sp*	92%
C015	Partial	*Bacillus safensis*	97%
C016	Partial	*Arthrobacter sp*	91%
C020	Partial	*Paenibacillus glebae*	97%
C024	Partial	unidentified	-
C031	Partial	*Bacillus safensis*	99%
C032	Partial	*Bacillus safensis*	91%
C033	Partial	*Paenibacillus pabuli*	98%
C054	Partial	*Bacillus cereus*	93%
C058	Partial	*Pseudomonas sp.*	86%
C059	Partial	unidentified	-
C060	Partial	*Bacillus safensis*	98%
C066	Partial	unidentified	-
C080	Partial	*Bacillus amyloliquefaciens*	98%
C082	Partial	unidentified	-
C083	Partial	*Paenibacillus terrigena*	99%
C084	Partial	unidentified	-
C090	Partial	*Bacillus safensis*	99%
C091	Partial	unidentified	-
C101	Partial	*Bacillus sp.*	99%
C109	Partial	unidentified	-
C116	Partial	*Lysinibacillus fusiformes*	99%
C120	Partial	unidentified	-
C129	Partial	unidentified	-
C131	Partial	unidentified	-
C136	Partial	unidentified	-
C143	Partial	unidentified	-
C153	Partial	unidentified	-
C154	Partial	unidentified	-
C155	Partial	unidentified	-
C158	Partial	unidentified	-
C161	Partial	unidentified	-
C169	Partial	unidentified	-
C177	Partial	unidentified	-
C180	Partial	*Streptomyces herbaceus*	92%
C185	Partial	*Bacillus velezensis*	93%
C188	Partial	*Streptomyces atratus*	94%
C195	Partial	*Bacillus vallismortis*	98%
C201	Partial	*Bacillus amyloliquefaciens*	97%
C203	Partial	*Bacillus safensis*	96%
C210	Partial	*Bacillus pumilus*	97%
C213	Partial	*Lysinibacillus xylanilyticus*	97%
C215	Partial	*Bacillus sp*	97%

continue

C217	Partial	*Bacillus cereus*	98%
C222	Partial	*Bacillus sp.*	97%
C226	Partial	*Lysinibacillus fusiformis*	98%

4.4 Discussion

Several modifications in culture media composition can allow isolation of "unculturable" microorganisms as previously reported (VARTOUKIAN et al., 2010; STEWART, 2012). The use of micronutrients like iron (Fe), copper (Cu), zinc (Zn) and molybdenum (Mo) for specific enzymes related to nitrogen fixing and denitrifiers (GLASS and ORPHAN, 2012) under hypoxic condition retrieve great number of potential denitrifying bacteria combined to cellulose in media for potential cellulose degraders.

However, few isolates presented genes for xylan degradation (*GH10* and *GH11* genes) that was present in T50 and T80 from the metatranscriptome of maize stover degradation (Study II); and these times of degradation matches to the end of Study I experiment. With this result, the primers set would be not appropriated to amplify the xylan genes in these strains and in addition other genes for degradation of carbon sources would be tested for a better characterization of the isolates; following the CAZymes database (CANTAREL et al., 2009) specialized in carbohydrate enzymes.

Positively, the identification of isolates showed most of strains identification belonging to Bacilli class as known cellulose and hemicellulose degrader with multi-enzyme complexes in special species like *B. subtilis*, *B. amyloliquefaciens* and *B. licheniforms* (LEE et al 2008; JONES et al., 2012; KIM et al., 2012; AMORE et al., 2013; DI PASQUA et al., 2014). And all other isolates obtained are lignocellulolytic as well (BUI, 2014; LÓPEZ-MONDÉJAR et al., 2016; VENTORINO et al., 2016). Moreover, all isolates belong to the same taxonomic Classes prospected in maize rhizosphere as bioindicators for GHGs mitigation under maize stover coverage in previous studies (Studies I and II).

4.5 Conclusion

Finally, modified culture media under low oxygen concentration (2% O_2) for long period of incubation allowed microorganisms with slow growth capabilities to arise and be isolated. Several strains have genes for nitrogen cycle (*nifH* and *nosZ*) and some for glycoside hydrolases for xylan (families GH10 and GH11). However, other genes could be analyzed for a robust analysis of these strains, for better characterization. And a complete sequencing of taxonomic genes or full genome could be interesting depending on their activities.

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