OSNAR OBEDE DA SILVA ARAGÃO

THE INFLUENCE OF STORAGE TIME ON SOIL MICROBIOLOGICAL ATTRIBUTES AND THEIR EFFECTIVENESS IN MONITORING SOIL QUALITY IN COFFEE CULTURE

LAVRAS - MG
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Coorientadores

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À minha mãe Maria Mirtes Lopes da Silva, expressão maior do meu amor e carinho, que me deu todo o apoio e força para continuar sonhando.

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DEDICO ESTA TESE

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RESUMO GERAL

Garantir a qualidade dos solos agrícolas é fundamental para a produção sustentada de alimentos. Um solo de alta qualidade possui valores de atributos microbiológicos satisfatórios. Esses atributos são preditores precoces e eficazes das mudanças que ocorrem no solo. Mas seus valores como indicador podem ser influenciados pela metodologia utilizada e classe de solo avaliada. Os objetivos desta tese foram analisar o efeito do tempo de armazenamento e de diferentes classes de solos sobre os principais atributos microbiológicos utilizados como indicadores de qualidade do solo e o potencial desses atributos como indicadores da qualidade de diferentes solos sob lavouras de café. Sete atributos foram avaliados (carbono de biomassa microbiana, respiração microbiana basal, hidrólise de diacetato de fluoresceína - FDA, e a atividade das enzimas urease, β-glucosidase, arilsulfatase e fosfatase ácida) em cinco tempos de armazenamento diferentes (15 horas e 15, 30, 60 e 120 dias). As amostras de solo foram provenientes de quatro ambientes distintos (Latossolo sob floresta, Latossolo sob café, Planossolo sob floresta e Planossolo sob café). Dependendo do tempo de armazenamento, todos os atributos foram capazes de diferenciar os ambientes. Longos tempos de armazenamento comprometem o uso da maioria dos atributos como indicadores de qualidade do solo. Portanto, devem ser considerados quando esses atributos forem usados como indicadores de qualidade do solo. Diferentes classes de solo sob condições que permitem acumular matéria orgânica, podem ter valores equiparáveis de carbono da biomassa microbiana, respiração basal microbiana, urease e qCO_2. O carbono da biomassa microbiana, FDA, urease, β-glucosidase e fosfatase ácida exibiram maior capacidade de discriminação das áreas mostrando que esses atributos são bons indicadores da qualidade dos solos cultivados com café.

Palavras-chave: Indicadores microbiológicos. Enzimas do solo. Carbono da biomassa microbiana. Armazenamento do solo. *Coffea arabica* L.. Mata Atlântica.

GENERAL ABSTRACT

Ensuring the quality of agricultural soils is essential for the sustainable production of food. A high-quality soil has satisfactory microbiological attribute values. These attributes are early and effective predictors of soil changes. But their values as an indicator can be influenced by the methodology used and the soil class evaluated. The objective of this thesis was to analyze the effect of storage time and different soil classes on the main microbiological attributes used as indicators of soil quality and the potential of these attributes as indicators of the quality of different soils under coffee crops. Seven attributes were evaluated (microbial biomass carbon, basal microbial respiration, fluorescein diacetate hydrolysis - FDA, and the activity of the enzymes urease, β-glucosidase, arylsulfatase, and acid phosphatase) in five different storage times (15 hours and 15 hours, 30, 60 and 120 days). The soil samples came from four different environments (Oxisol under forest, Oxisol under coffee, Planosol under forest, and Planosol under coffee). Depending on the storage time, all attributes were able to differentiate the environments. Long storage times compromise the use of most attributes as indicators of soil quality. Therefore, they should be considered when these attributes are used as indicators of soil quality. Different soil classes under conditions that allow organic matter to accumulate may have values comparable to microbial biomass carbon, microbial basal respiration, urease, and qCO2. The carbon of the microbial biomass, FDA, urease, β-glucosidase, and acid phosphatase exhibited greater capacity to discriminate areas showing that these attributes are good indicators of the quality of soils cultivated with coffee.

Keywords: Microbiological indicators, Soil enzymes, Microbial biomass carbon, Soil storage, *Coffea arabica* L., Mata Atlântica.

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LISTA DE ABREVIATURAS, SIGLAS E FÓRMULAS QUÍMICAS

Ag_2SO_4 - Sulfato de prata

Aril - Enzima Arilsulfatase

β-glu - Enzima β-glucosidade

$CaCl_2$ - Cloreto de potássio

$CaCO_3$ - Carbonato de cálcio

$CHCl_3$ - Clorofórmio

CO_2 - Dióxido de carbono

CBM - Carbono da biomassa microbiana

FDA - Hidrólise do diacetato de fluoresceína

H_2SO_4 - Ácido sulfúrico

H_3PO_4 - Ácido fosfórico

HCl - Ácido Clorídrico

$K_2Cr_2O_7$ - Dicromato de potássio

K_2SO_4 - Sulfato de potássio

KCl - Cloreto de potássio

MO - Matéria orgânica do solo

NaOH - Hidróxido de sódio

NH_3 - Amônia

NH_3 - Amônio

nm - Nanômetro

NPK - Nitrogênio fósforo e potássio

pH - Potencial de hidrogeniônico

PNF - p-nitrofenol

PNG - p-nitrofenil-b-D-glucosideo

PNS - p-nitrofenol sulfato

qCO_2 - Quociente metabólico

*q*MIC – Quociente microbiano

rpm - Rotação por minuto

RB - Respiração microbiana basal

SB - Soma de bases

THAM - Tris hidroximetil aminometano

SUMÁRIO

PRIMEIRA PARTE (Capítulo I)

1 INTRODUÇÃO GERAL

O solo é fundamental para a manutenção da vida no planeta. Sua importância abrange desde o fornecimento de energia para as cadeias tróficas até a prestação de serviços ecossistêmicos globais. Por ser um recurso natural formado a uma taxa muito lenta (370 a 1290 kg ha^{-1} ano^{-1})* e que pode ter vários centímetros destruídos em uma única chuva, é imperiosa a necessidade de se manejar esse ecossistema adequadamente. Considerando sua importância, mais de 14 mil trabalhos científicos foram publicados desde o ano de 1940 tratando do tema qualidade do solo (BASTIDA et al., 2008). Com isso os avanços nas pesquisas sobre os subsistemas físicos e químicos do solo foram expressivos. Entretanto relativamente pouco se estudou sobre o subsistema biológico. Em razão da maior necessidade de preservação do solo, no início da década de 1990 o tema qualidade do solo passou a ser de interesse global. Após esse período, o subsistema biológico do solo começou ser estudado como componente indissociável do funcionamento harmonioso desse ecossistema. Em 2015 o tema foi reaquecido na reunião das Nações Unidas em Paris que destacou a qualidade do solo como uma das metas fundamentais do objetivo "agricultura sustentável" (ONU, 2015).

O interesse global pela qualidade do solo incentivou as publicações de muitos trabalhos que utilizaram os atributos microbiológicos como indicadores da qualidade do solo. Os atributos mais utilizados nesses trabalhos foram: carbono da biomassa microbiana, respiração basal microbiana, quociente metabólico, hidrólise do diacetato de fluoresceína e atividades enzimáticas (GIL-SOTRES, 2005). Entre as enzimas, as mais utilizadas foram: β-glucosidase, urease, fosfatase e arilsulfatase, devido suas relações com os biociclos do carbono, nitrogênio, fósforo e enxofre, respectivamente (GIL-SOTRES, 2005). Além disso essas enzimas atendem a maioria das premissas de um bom indicador, isto é, apresentam intrínseca relação com funções chaves do solo, são sensíveis ao maior número possível de agentes contaminantes, são de fácil avaliação e refletem níveis diferentes de degradação do solo.

Os resultados publicados sobre esses atributos são contrastantes e muitas vezes incongruentes entre diferentes solos ou mesmo para a mesma classe de solo. As razões

*Estimativa global baseada no balanço geoquímico das rochas, solos e água (Wakatsuki e Rasyidin, 1992)

mais prováveis para esses contrastes podem estar associadas, em parte, à complexidade do solo, heterogeneidades dos ambientes e características intrínsecas dos atributos. Nesse caso, o maior desafio é identificar os atributos microbiológicos mais consistentes em detectar mudanças na qualidade do solo em função de práticas de manejo. Nesse sentido, o qCO_2 é um bom exemplo, pois frequentemente se associa a ambientes que apresentam algum grau de estresse ecológico (ANDERSON; DOMSCH, 1985). Para os demais atributos microbiológicos, as respostas divergentes sob mesmas condições edáficas são intrigantes e alertam para a possibilidade da influência dos procedimentos metodológicos nesses resultados. Entre esses procedimentos, as distintas formas e épocas de coleta, pré-tratamento das amostras, concentração do substrato, temperatura e tempo de armazenamento são as principais razões metodológicas para a origem de resultados contraditórios nos estudos (GIL-SOTRES, 2005). Em relação à profundidade de coleta, uma parte significativa das publicações têm utilizado a profundidade de 0-10 cm, isso pode ser um indicativo da existência de um consenso em relação a esse aspecto. Não obstante, esse é um procedimento que precisa ser padronizado nas metodologias pois outras profundidades (0-5, 0-15, 0-20, 0-30 cm) também aparecem na literatura (BOWLES et al., 2014; MAYOR et al., 2016; BISWAS et al., 2017; DILLY et al., 2018). Para a época de coleta, a literatura disponível mostra que existe um efeito nas respostas dos atributos entre os períodos secos e chuvosos. Entretanto esse efeito (se melhor ou pior) varia entre os trabalhos. De modo que tanto a profundidade quanto a época de coleta precisam ser relatadas nos trabalhos para que sejam comparáveis entre si. A concentração dos substratos foi adequadamente discutida por DeForest (2009). Por outro lado, temperatura de armazenamento a 4ºC embora pareça estar bastante corroborada (LEE et al., 2007; DEFOREST, 2009), pois minimiza a morte microbiana e a inativação enzimática (ROSS, 1965; ANDERSON; DOMSCH, 1993), tem sido recentemente discutida no conceito FERTBIO por pesquisadores brasileiros (LOPES et al., 2015; MENDES et al., 2019). Entre esses aspectos metodológicos o tempo de armazenamento é o menos considerado pelos cientistas dessa área. Embora muitos autores relatem que as análises devam ser efetuadas o mais rápido possível, nem sempre isso é possível devido a logística, a distância dos locais de coleta, a disponibilidade de mão-de-obra e as limitações de espaços nos laboratórios. Isso implica, por muitas vezes, na necessidade de longos tempos de armazenamento. Ao se considerar que o tempo de armazenamento pode influenciar de forma decisiva na eficácia dos atributos microbiológicos como indicadores de qualidade, este trabalho de tese se propõe: verificar a influência do tempo de

armazenamento nos principais atributos microbiológicos utilizados como indicadores da qualidade do solo e verificar a influência de diferentes classes de solos e coberturas vegetais (sistemas em clímax e agrícolas) nos atributos microbiológicos e o potencial desses atributos como indicadores da qualidade de diferentes solos sob lavouras de café convencional no domínio da Mata Atlântica.

2 REVISÃO DE LITERATURA E FUNDAMENTAÇÃO TEÓRICA

2.1 Qualidade do solo

O conceito qualidade do solo é muito variável e muitas vezes arbitrário. Inclui desde a capacidade produtiva para um viés agrícola até aspectos relacionados ao meio ambiente (BASTIDA et al., 2008). Doran e Parkin, (1994) definiram qualidade do solo como sendo "*a capacidade de um solo funcionar dentro dos limites do ecossistema e do uso da terra para sustentar a produtividade biológica, manter a qualidade ambiental e promover a saúde animal e vegetal*". Karlen et al. (1997) propuseram uma definição que difere a de Doran e Parkin (1994) apenas por enfatizar as particularidades do solo, isto é, "*qualidade do solo é capacidade de um solo específico em prestar serviços ecossistêmicos"* Esses conceitos, especialmente o primeiro, foram alvos de fortes críticas de cientistas dessa área (SOJKA; UPCHURCH, 1999; LETEY et al., 2003). As críticas abordavam diversos aspectos (BÜNEMANN et al., 2018), mas o principal deles foi à forma original da definição que exibiu muita ênfase e valor a um número limitado de culturas anuais que fornecem alimentos baratos e são fortemente subsidiadas. Provavelmente o conceito proposto por Doran e Parkin (1994) tenha sofrido influência do conceito mais antigo proposto por Mausel (1971) que definiu a qualidade do solo como "*a capacidade dos solos de produzir milho, soja e trigo em condições de manejo de alto nível"*. Com essas críticas, vários conceitos foram propostos e eles consideraram o fato de que o solo pode ser usado para uma variedade maior de propósitos (NORTCLIFF, 2002). Dessa forma, além dos aspectos relacionados ao uso do solo, que remetem à ideia da necessidade de intervenção humana para obtenção de resultados, a capacidade do solo em fornecer serviços ambientais foi igualmente enfatizada.

No contexto de uso, qualidade é frequentemente relacionada a capacidade produtiva do solo (MAUSEL, 1971; ARAGÃO et al., 2020) enquanto que no contexto ambiental, o conceito qualidade é associado a capacidade do solo em contribuir com os serviços ecossistêmicos e a preservação da biodiversidade dos ecossistemas naturais (BÜNEMANN et al., 2018). Essa amplitude conceitual dificulta o ranqueamento das

características capazes de indicar um solo em seu estado ideal. Apesar da dificuldade em se estabelecer um conceito suficientemente capaz de definir a qualidade do solo, sabe-se que um solo com qualidade (saudável) é fundamental para a manutenção da vida no planeta. As críticas levaram a uma discussão que colocou o manejo como uma questão central da qualidade do solo (BÜNEMANN et al., 2018). Sojka e Upchurch (1999) também ressaltaram o manejo como cerne da discussão e propuseram maior foco ao uso do solo do que em suas funções. Dessa forma, a responsabilidade de se manter a qualidade desse ambiente seria mais atribuída aos seus usuários. Nesse sentido, agricultores, administradores de áreas de conservação, margens de estradas, cursos de água, áreas verdes urbanas, todas as indústrias e todos aqueles que dependem do solo direta e indiretamente precisariam ter participação na manutenção da qualidade do solo (BÜNEMANN et al., 2018). Nessa perspectiva, Bastida et al. (2008) sugeriram que o mais importante seria a aplicação dos conceitos de qualidade já existentes, o que aceleraria a elaboração de políticas de gestão da qualidade do solo.

No sentido de tornar mais pragmático o conceito qualidade do solo e aplicá-lo para as condições tropicais, pode-se dizer que que um solo com qualidade é aquele em que a relação entre os processos ordenativos e os processos dissipativos é > 1. Nesse cenário, as práticas que contribuem para a adição e manutenção da matéria orgânica estão superando as práticas que promovem processos de perda (processos dissipativos). Um solo sob essas condições, provavelmente terá sua qualidade sustentada ao longo do tempo. Por outro lado, aceitar esse conceito no seu extremo pode levar à entendimentos equivocados. Por exemplo, o de que um Organossolo com caráter tiomórfico possui elevada qualidade, o que não é verdade devido a abundância de enxofre e seus derivados. Isso mostra que pressupostos muito rígidos podem contribuir para a indefinição da qualidade do solo, ao invés de facilitar o entendimento do conceito.

2.2 Indicadores de qualidade do solo

"*Um indicador de qualidade do solo é uma propriedade mensurável que influencia a capacidade de um solo em desempenhar uma determinada função*" (ACTON; PADBURY, 1993). Os indicadores mais comuns são matéria orgânica, pH, P disponível e armazenamento de água (BÜNEMANN et al., 2018). Embora esse cenário esteja mudando gradativamente, os atributos biológicos e/ou microbiológicos ainda aparecem sub representados nos trabalhos com esse objetivo. Todavia, por responderem mais rapidamente às mudanças que ocorrem no solo, precisam ser mais explorados. Os

atributos microbiológicos mais utilizados nos trabalhos sobre qualidade do solo são aqueles relacionadas aos biociclos do Carbono, Nitrogênio, Fósforo e Enxofre (GIL-SOTRES, 2005). Esses atributos estão descritos detalhadamente a seguir.

Carbono da Biomassa Microbiana

A biomassa microbiana do solo é a fração ativa da matéria orgânica. Ela é constituída principalmente por organismos dos reinos Archea, Bactéria, Fungi, protoctistas e nematóides do reino Animália. Os grupos mais representativos da biomassa microbiana são os fungos, bactérias, actinobactérias, leveduras e protozoários (MOREIRA; SIQUEIRA, 2006). Embora essa fração raramente ultrapasse 5% do total de matéria orgânica do solo (SMITH; PAUL, 1990), sua função é imprescindível nesse sistema. Ela é a principal fonte de enzimas no solo e por isso é responsável pela maior parte das transformações orgânicas e geração de energia para as cadeias tróficas superiores. Entre os métodos de quantificação da biomassa microbiana o carbono extraído das amostras é muito utilizado pelos pesquisadores dessa área. O método consiste na exposição das amostras ao clorofórmio e, o carbono liberado pela morte dos microrganismos é determinado por extração, oxidação e digestão química seguida de titulação. Cada 1 ml de $K_2Cr_2O_7$ que não reage com o carbono extraído pelo K_2SO_4 equivale à 1.200 µg de carbono da biomassa microbiana (VANCE et al., 1987).

O Carbono microbiano tem uma relação direta com a quantidade e qualidade da matéria orgânica do solo por isso é um importante indicador de qualidade. Na literatura foi relatada uma redução de 40% do carbono da biomassa microbiana quando sistemas naturais foram convertidos em agricultura itinerante na Amazônia (VILLANI et al., 2017). Por outro lado, em sistemas de plantio direto foi verificado aumento de até 103% nesse atributo (BALOTA et al., 1996; SANTOS et al., 2015). Carneiro et al. (2009) e Melloni et al. (2017) observaram efeitos positivos à microbiota naqueles sistemas onde havia a presença de gramíneas nas entrelinhas das culturas. Redução das comunidades microbianas em sistemas com uso excessivo de agroquímicos, fogo e pastagens sobrecarregadas também foi relatada na literatura (KASCHUK et al., 2010). Todos esses relatos mostram que a biomassa microbiana do solo é dependente da quantidade e qualidade da matéria orgânica e práticas conservacionistas de uso do solo.

Respiração Basal Microbiana

A respiração microbiana é um parâmetro utilizado para quantificar a atividade dos microrganismos do solo. A atividade microbiana é a expressão de todas as reações bioquímicas catalisadas pelos microrganismos, principalmente por fungos e bactérias (ALVAREZ et al., 1995). Quando os microrganismos oxidam a matéria orgânica eles utilizam o O_2 e produzem CO_2. Portanto a respiração basal microbiana é medida pela quantidade de CO_2 produzido durante a oxidação da matéria orgânica do solo (JENKINSON; POWLSON, 1976; ALEF; NANNIPIERI, 1995). O aumento da respiração microbiana pode ocorrer tanto pela maior quantidade de matéria orgânica depositada ao solo, quanto pela resposta à uma condição de estresse imposta ao ambiente. Na literatura essas duas situações são frequentemente relatadas (EVANGELISTA et al., 2013; LOPES et al., 2013) e revelam que a respiração microbiana não é, por si só, suficientemente elucidativa das condições do solo. Entretanto sua quantificação é fundamental (ARAGÃO et al., 2020), pois permite calcular o quociente metabólico - qCO_2 (ANDERSON; DOMSCH, 1993).

Quociente metabólico (qCO_2) e quociente microbiano (qMIC)

O qCO_2 e qMIC são parâmetros microbiológicos indiretos, calculados a partir da relação entre diferentes atributos do solo. O qCO_2 também referido na literatura como respiração microbiana específica (PIRT, 1975) contribui para o entendimento dos processos ordenativos ou dissipativos no solo. O qCO_2 é resultante da razão entre a atividade microbiana e o carbono da biomassa microbiana (ANDERSON; DOMSCH, 1993). Originalmente foi expresso em ng $C\text{-}CO_2$ µg C_{mic} h^{-1}, mas em decorrência da biomassa e a respiração serem frequentemente expressas em mg por g de solo dia^{-1}, o qCO_2 tem sido expresso em mg $C\text{-}CO_2$ g^{-1} MBC dia^{-1}.

Quando uma grande unidade de biomassa microbiana está respirando mais, significa que o aporte de matéria orgânica ao solo está sendo constante, nesse caso o qCO_2 é menor. Entretanto quando uma pequena unidade de biomassa está respirando mais, significa que a oferta de matéria orgânica é baixa e as comunidades estão degradando compostos mais recalcitrantes do solo e por isso gastando mais energia. Nesse último caso, o qCO_2 é maior e pode indicar uma condição de estresse ecológico da comunidade microbiana. Como o qCO_2 é um atributo muito utilizado na avaliação da qualidade do solo, um resumo da sua importância foi adequadamente apresentado na literatura (BASTIDA et al., 2008).

O *q*MIC é o resultado da relação entre o carbono da biomassa microbiana e o carbono orgânico total (SPARLING, 1992), é expresso em % e contribui para o entendimento da qualidade da matéria orgânica. Sob condições de deposição de compostos orgânicos facilmente decomponíveis, especialmente aqueles com predomínio de radicais do grupo o-alquil, a biomassa microbiana aumenta a uma taxa maior que o carbono orgânico total. Nesse caso o *q*MIC é maior e indica aumento do número de células microbianas em decorrência da maior energia disponível. Por ser mais sensível, a biomassa microbiana tem uma tendência de variar mais quando comparada ao carbono orgânico total, por isso, o resultado dessa relação é muito variável no tempo e no espaço (INSAM; DOMSCH, 1988). Contudo, em condições de equilíbrio ecológico o *q*MIC tende a estabilidade.

Hidrólise do diacetato de fluoresceína (FDA)

A hidrólise do diacetato de fluoresceína não é uma enzima ou um grupo de enzimas do solo, é um substrato (3', 6'-Diacetylfluorescein) sintético que pode ser hidrolisado por diversas classes de enzimas do solo como as proteases, lipases e esterases (GUILBAULT et al., 1964). Quando esse substrato é hidrolisado o produto de sua transformação é a fluoresceína. Esta pode ser visualizada dentro das células por microscopia de fluorescência. Essa fluorescência pode ser quantificada por fluorimetria ou espectrofotometria (SCHNURER; ROSSWALL, 1982; DICK, 1996). Por ser hidrolisado por diversas enzimas, o diacetato de fluoresceína é utilizado para expressar a atividade enzimática global do solo. Além disso, é também usado para determinar a atividade microbiana total no solo (SWISHER; CARROLL, 1980). A FDA apresenta relação positiva com a respiração microbiana do solo (FROUZ; NOVÁKOVÁ, 2005) e por ser sensível em detectar diferentes formas de uso é frequentemente usada como indicador da qualidade do solo (TRANNIN et al., 2007; CARNEIRO et al., 2009; ARAGÃO et al., 2020).

Enzima urease

A urease ou ureia amida hidrolase é uma enzima da classe das hidrolases que rompem ligações químicas funcionais por hidrólise (TABATABAI; BREMNER, 1972; BURNS, 1978). Essa enzima atua na hidrólise da ureia, transformando-a em amônia e dióxido de carbono (BYRNES; AMBERGER, 1989). Entre as enzimas do solo, a urease é uma das mais estudadas devido sua importância na regulação do fornecimento de

nitrogênio às plantas. Em razão da dinâmica do nitrogênio no solo, existe sempre um dilema sobre o nível adequado de atividade dessa enzima nos solos agrícolas. Se por um lado, alta atividade significaria maior disponibilidade de nitrogênio assimilável pelas plantas, por outro lado, o aumento do nitrogênio disponível no solo pode resultar em perdas por volatilização (amônia) ou por lixiviação (amônio), dependendo das condições ambientais predominantes (BALOTA et al., 2013).

A urease possui estreita relação com a textura do solo. Dependendo da proporção entre as frações granulométricas do solo, essa enzima pode persistir por maior ou menor tempo nesse ambiente. A razão para a maior estabilidade em solos mais argilosos está associada a predominância de "microambientes" que protegem a urease da biodegradação por enzimas proteolíticas (BORGHETTI et al., 2003). Além disso, a urease pode se complexar por ligações covalentes aos coloides orgânicos e permanecer no solo em forma de complexos húmico-proteicos (ZANTUA; BREMNER, 1977). Assim, manejos que aumentam o teor de matéria orgânica são significativamente favoráveis à manutenção da urease no solo. A manutenção dessa enzima é fundamental para o biociclo do nitrogênio e ela tem sido apresentada na literatura como um importante indicador da qualidade, por ser sensível a diferentes formas de uso do solo (ROLDÁN et al., 2005; LANNA et al., 2010; LISBOA et al., 2012) e está associada com solos mais produtivos (ARAGÃO et al., 2020)

β-glucosidase

A β-glucosidase é uma subclasse de enzimas da classe das hidrolases (BURNS, 1978). Essa subclasse é muito abundante no solo e sua denominação β está relacionada com o tipo de ligação que a enzima hidrolisa. A β-glucosidase, portanto, hidrolisa a ligação glicosídica â-1,4 de um dissacarídeo transformando-o em celobiose. Esse é o processo limite da transformação da celulose em glicose. O processo completo dessa transformação é mediado por diversas enzimas celulolíticas e está adequadamente descrito em Saha et al. (1994). Nesse processo, a β-glucosidase ganha destaque não apenas por mediar uma etapa imprescindível da transformação, mas também por reduzir a inibição da celobiose às demais enzimas, garantindo que elas funcionem de modo mais eficiente.

Considerando a importância da glicose como fonte de energia, a abundância dessa enzima no solo é fundamental para o adequado funcionamento desse ecossistema (TABATABAI, 1994). Por estar associada às transformações dos resíduos orgânicos, a

β-glucosidase reflete a dinâmica do carbono e a atividade biológica do solo. Por ser sensível às mudanças que ocorrem no solo, essa enzima é frequentemente usada para monitorar sua qualidade (BANDICK; DICK, 1999; ROLDÁN et al., 2005; LISBOA et al., 2012; SANTOS et al., 2015; ARAGÃO et al., 2020).

Fosfatase

O termo fosfatase refere-se a um grupo de enzimas que hidrolisam éster e anidridos de fosfato. As fosfatases atuam na transformação do fósforo orgânico à fósforo inorgânico (PO_4). A comissão de enzimas da Sociedade Bioquímica Internacional propôs uma divisão dessas enzimas em subgrupos de acordo com o tipo de compostos que hidrolisam (FLORKIN; STOTZ, 1964). Dessa forma, cinco subgrupos foram criados (fosfomonoesterases, fosfodiesterases, fosfotriesterases, metafosfatases e pirofosfatases). No subgrupo das fosfomonoesterases estão as enzimas fitase, nucleotidase, açúcar fosfatase e glicerofosfatase. As nucleases e fosfolipases compõem o subgrupo das fosfodiesterases que degradam ácidos nucleicos e fosfolipídios, respectivamente (FLORKIN; STOTZ, 1964; EIVAZI; TABATABAI, 1977). Enquanto as fosfotriesterases degradam grupos fosforil (FLORKIN; STOTZ, 1964; EIVAZI; TABATABAI, 1977). Entre as fosfatases, as fosfomonoesterases são as mais conhecidas e incluem a ortofosfórica monoéster fosfohidrolase EC 3.1.3.2 conhecida como fosfatase ácida e a ortofosfórica monoéster fosfohidrolase EC 3.1.3.1, conhecida como fosfatase alcalina. Essas enzimas se diferenciam somente pelo último código que recebem da Comissão de Enzimas que representa a mais específica reação catalisada por elas (TABATABAI; BREMNER, 1969; EIVAZI; TABATABAI, 1977; DICK et al., 1996). As fosfatases são fundamentais para o funcionamento do solo pois atuam na mineralização do fósforo orgânico e consequente disponibilização às plantas. Por apresentarem estreita relação com a matéria orgânica do solo, essas enzimas são frequentemente usadas como indicadoras da qualidade do solo (GIL-SOTRES, 2005).

Arilsulfatase

Arilsulfatase é a terminologia que designa um grupo de enzimas que hidrolisam ésteres de sulfato orgânico resultando em produção de sulfato (SO_4), forma de enxofre assimilável pelas plantas (TABATABAI; BREMNER, 1970). Por hidrolisar a mais abundante forma de enxofre do solo (ésteres de sulfato [40-70% do S do solo]), a arilsulfatase sulfohidrolase EC 3.1.6.1. é a enzima mais estudada desse grupo

(TABATABAI, 1994). A presença abundante dessa enzima no solo é um indicativo de alta taxa de mineralização do enxofre orgânico e pode indicar alta biomassa de fungos, que são os principais produtores de ésteres de sulfato do solo (DICK et al., 1996). A arilsulfatase, por discriminar diferentes manejos e/ou formas de uso do solo, é apresentada na literatura como importante atributo indicador da qualidade do solo (BANDICK et al., 1994; BANDICK; DICK, 1999; LISBOA et al., 2012; MENDES et al., 2019).

2.3 Atributos microbiológicos em solos de florestas e em áreas agrícolas no domínio da Mata Atlântica

A Mata Atlântica é um dos biomas brasileiros mais ricos em biodiversidade. A área de domínio desse bioma se estende ao longo da costa litorânea brasileira que vai do Rio Grande do Norte ao Rio Grande do Sul. Originalmente a área de domínio da Mata Atlântica abrangia 47 % do Estado de Minas Gerais, no entanto, apesar de ser o estado com maior área remanescente da floresta, restam apenas 10,2 % desse total (FUNDAÇÃO SOS MATA ATLÂNTICA, 2018). Pela sexta vez o Estado lidera o ranking de maior desmatador da Mata Atlântica. Foram destruídos 3.379 hectares da vegetação entre os anos de 2017 e 2018 (FUNDAÇÃO SOS MATA ATLÂNTICA, 2018). Em razão da importância desse bioma e da necessidade de sua preservação, muitas informações foram geradas sobre seu componente vegetal e animal (TABARELLI et al., 2005). No entanto, pouco se sabe sobre o impacto no funcionamento microbiológico do solo quando essa floresta é convertida em sistemas agrícolas. O conhecimento produzido até agora, deriva principalmente de estudos com pastagens e culturas que requerem preparos periódicos do solo. A maioria desses estudos avaliou atributos microbiológicos gerais como carbono, nitrogênio e respiração microbiana. Para esses atributos, alguns estudos mostraram superioridade dos solos da floresta comparados aos solos de culturas anuais (SILVA et al., 2012; ALMEIDA; SILVA, 2016). Essa superioridade, no entanto, não foi observada para sistemas agroflorestais e áreas de pastagem (SILVA et al., 2012; NOGUEIRA et al., 2016; TAVARES et al., 2018).

Em solos cultivados com café no domínio da Mata Atlântica, os resultados para os atributos microbiológicos são divergentes. Existem relatos de que a utilização de leguminosas como adubo verde influencia a atividade microbiana, tanto sob a copa do café quanto entre as fileiras (BALOTA; CHAVES, 2010). Por outro lado, não houve diferença nos valores desses atributos em áreas com ou sem crotalária (PIMENTEL et al., 2006). Lammel et al. (2015) ao avaliarem a influência de diferentes sistemas de manejo

do café nos parâmetros biológicos no sul de Minas Gerais, relataram que o uso combinado de todos os atributos foi necessário para diferenciar os quatro sistemas de cultivo avaliados (convencional, orgânico e consorciado com *Brachiaria decumbens* e *Arachis pintoi* na entrelinha). Neste sentido, Partelli et al. (2012) observaram os melhores índices de qualidade do solo na Mata Atlântica e café sob manejo orgânico. Em contraste, não foi observada diferenças significativas entre as áreas de Mata Atlântica e café arábica sob manejo orgânico e convencional para atributos como respiração e quociente microbiano (THEODORO et al., 2003; PIMENTEL et al., 2011). A omissão de informações sobre os procedimentos metodológicos na maioria desses trabalhos não permite uma análise sistêmica sobre essas divergências. É possível que elas estejam relacionadas com as especificidades dos ambientes e atributos avaliados, mas podem também ser decorrentes da ausência de padronização nos procedimentos metodológicos.

2.4 Classes de solo de diferentes texturas e as comunidades microbianas e enzimas do solo

O efeito da textura do solo na dinâmica dos microrganismos é bastante controverso nas publicações. Espera-se que os solos com menores granulometrias (argilosos e siltosos) favoreçam as comunidades microbianas. Entretanto a interação entre textura e microrganismos é complexa e os resultados disponíveis na literatura não são suficientemente elucidativos desse aspecto. Por um lado, os solos com predomínio de argila e silte podem favorecer as comunidades microbianas pela proteção física contra predação pela mesofauna e influência de fatores abióticos. Por outro lado, esses mesmos solos podem impor restrições aos microrganismos por tornarem a matéria orgânica menos acessível à decomposição (VAN VEEN, 1987). Provavelmente por esse motivo a relação positiva dos solos de texturas mais finas e a comunidade microbiana nem sempre é observada (WARDLE, 1992). A maioria do conhecimento disponível sobre esse tema foi produzido a partir de solos agrícolas frequentemente perturbados (BAUHUS, 1999). Talvez por isso, o conhecimento comum tratado no início desse tópico sobre a relação positiva das texturas mais finas com a comunidade microbiana, seja tão difundido. Entretanto, classes de solos de diferentes texturas podem exibir resultados diferentes sob ambientes florestais. Isso porque, a floresta é um ecossistema sob maior equilíbrio ecológico, cujo o aporte constante de resíduos orgânicos pode reduzir os efeitos da textura nos microrganismos. Com conhecimento disponível, é possível inferir que a ciclagem da biomassa microbiana é maior nos solos mais grosseiros (VAN VEEN, 1987; HASSINK,

1994; BECK et al., 1995). No entanto essa ciclagem parece estar mais associada com menor capacidade desses solos em proteger a matéria orgânica do que com interações diretas entre a comunidade microbiana e as frações minerais no tamanho areia. Provavelmente por isso, quando não há regularidade no fornecimento da matéria orgânica nesses solos, a biomassa microbiana reduz significativamente. Considerando que os microrganismos são a principal fonte de enzimas no solo, os fatos que interferem em sua sobrevivência, provavelmente interferirão na atividade enzimática. Nesse sentido, conhecer o comportamento microbiano em lavouras de café e florestas adjacentes formadas sobre classes de solos com diferentes texturas é fundamental para a seleção de atributos eficazes no monitoramento da qualidade ecológica desses ambientes.

2.5 Influência do tempo e condições de armazenamento do solo nos atributos microbiológicos

O tempo de armazenamento é um aspecto importante a ser considerado pelos cientistas que estudam os atributos microbiológicos como indicadores da qualidade do solo. Armazenamentos prologados podem reduzir os valores dos atributos e comprometer a qualidade dos dados gerados. Usualmente as amostras de solo são armazenadas a 4ºC (ROSS, 1965; ISO, 1993; LEE et al., 2007; DEFOREST, 2009; ABELLAN et al., 2011). O armazenamento nesta condição minimiza a morte microbiana e a inativação enzimática por preservar as condições originais do solo. Entretanto longos períodos sobre essa condição podem resultar em morte de células microbianas e/ou alteração da estrutura da molécula enzimática (LOPES, 2015). Por outro lado, o armazenamento em temperatura ambiente, recentemente proposto no conceito FERTBIO (LOPES et al., 2015; MENDES et al., 2019), reduz a atividade enzimática (LOPES et al., 2015). Isso ocorre devido as alterações dos componentes que contribuem para a atividade geral de uma enzima do solo (LADD, 1978). O armazenamento sob temperatura ambiente é considerado prático por alguns autores (LOPES et al., 2015; MENDES et al., 2019), pois permite a utilização dos mesmos solos das análises físicas e químicas. Isto seria interessante para os casos em que as amostras fossem imediatamente processadas (dentro de 24 horas), mas isso é impraticável na maioria das vezes. Do contrário, ainda não foi adequadamente elucidada a extensão dos efeitos dessa condição de armazenamento nas respostas dos atributos. As evidências que fundamentaram essa recomendação de armazenamento foram obtidas de amostras secas ao ar e armazenadas após um intervalo de dois anos (LOPES et al., 2015), não usual para esses tipos de análises. Além disso, a maior parte das publicações, não

recomenda o armazenamento em temperatura ambiente e secagem ao ar, pois esses tratamentos provocam reduções drásticas nos valores dos atributos (ROSS, 1965; TABATABAI; BREMNER, 1970, PANCHOLY; RICE, 1972, ANDERSON, 1987, PARHAM; DENG, 2000, LEE et al., 2007, WALLENIUS et al., 2010). Dessa maneira, trabalhos complementares são necessários para melhor compreensão desses efeitos.

Alguns autores mostraram que o congelamento das amostras mantém a atividade da maioria das enzimas e a estrutura da comunidade bacteriana relativamente estável (ROSS, 1965; TUNER; ROMERO, 2010; WALLENIUS, 2010). Entretanto essa condição de armazenamento é pouco prática por demandar muito espaço em freezers, tornando-se inviável em situações cujo volume de solo é grande.

Como alternativa, o armazenamento refrigerado a 4ºC é recomendado e amplamente utilizado em ensaios microbiológicos (ROSS, 1965; ISO, 1993; LEE et al., 2007; DEFOREST, 2009; ABELLAN et al., 2011). Esta temperatura de armazenamento está presente na grande maioria dos trabalhos sobre esse tema. Alef e Nannipieri, (1995) propuseram 4 semanas como um tempo razoável de armazenamento das amostras. Turner e Romero (2010) recomendaram duas semanas, enquanto na ISO10381-6, (2009) a recomendação é de 24 horas. Contudo não está claro nessas publicações o efeito do armazenamento em cada atributo, o que permitiria ao pesquisador adotar uma ordem de prioridade entre suas análises. Assim, é preciso considerar as particularidades de cada atributo microbiológico e fornecer informações mais práticas sobre esse tema. Informações nesse sentido podem ser muito úteis para os avanços dos estudos sobre a utilização dos atributos microbiológicos como indicadores da qualidade do solo. E esse é um dos objetivos deste trabalho de tese.

3 PROCEDIMENTOS METODOLÓGICOS

3.1 Análises microbiológicas

Nesta tese foram avaliados os seguintes atributos microbiológicos: carbono da biomassa microbiana, respiração basal microbiana, hidrólise do diacetato de fluoresceína e atividades das enzimas urease, β-glucosidade, fosfatase ácida e arilsulfatase.

Carbono de biomassa microbiana (MBC)

O carbono da biomassa microbiana foi determinado pelo método de fumigação e extração (VANCE et al., 1987). Vinte gramas de cada amostra de solo foram pesados em placas de petri (Figura 1a), com quatro repetições, duas delas foram fumigadas em um

dessecador contendo 30 ml de clorofórmio e duas não foram fumigados (Figura 1b). Após esse procedimento, todas as amostras foram incubadas no escuro a 27ºC por 24 horas. Para extração, 50 ml de K_2SO_4 (0,5 M) foram adicionados às amostras e a mistura foi agitada por 30 minutos. Na sequência, a suspensão foi filtrada em papel filtro nº 42 (Química Moderna). O carbono microbiano foi determinado pela digestão de 8 ml do extrato filtrado com 2 ml de $K_2Cr_2O_7$, 10 ml de H_2SO_4 (95%) e 5 ml de H_3PO_4 (85%). A mistura (Figura 1c) foi aquecida durante 5 minutos e, após o resfriamento, foi adicionado 10 ml de água destilada e titulado com sulfato ferroso amoniacal, utilizando difenilamina como indicador. Os mesmos procedimentos foram realizados nas amostras controle, exceto adição do extrato.

Respiração basal microbiana (MBR)

A estimativa do CO_2 evoluído durante a incubação consistiu em capturá-lo por NaOH e posterior titulação com HCl (JENKINSON; POWLSON, 1976). Alíquotas de 20 g de cada amostra de solo foram pesados e umedecidos até aproximadamente 55% da capacidade de campo e colocados em frascos hermeticamente fechados (Figura 1d), juntamente com 20 ml de uma solução de NaOH (0,5 M). A mistura foi incubada por 72 horas no escuro (Figura 1e). O controle foi formado por quatro recipientes com 20 ml de NaOH incubados nas mesmas condições, mas sem o solo. Após o período de incubação, o CO_2 capturado pelo NaOH foi precipitado pela adição de 5 ml de $BaCl_2.2\ H_2O$ (0,5 M). O NaOH que não reagiu foi titulado com HCl (0,5 M) e cinco gotas do indicador de fenolftaleína (0,1%) até mudar de cor vermelha incolor.

Figura 1 – Ilustração dos procedimentos metodológicos do carbono da biomassa microbiana e respiração basal microbiana. a) incubação do solo com clorofórmio, b) dessecador com amostras de solo fumigadas, c) amostras quantificadas do carbono da biomassa microbiana, d) solo incubado com NaOH e e) armazenado em ausência de luz.

Fonte: Arquivos do projeto do autor

Determinação da hidrólise do diacetato de fluoresceína (FDA)

A hidrólise do diacetato de fluoresceína por células microbianas foi determinada pela liberação de fluoresceína (DICK et al., 1996). O procedimento analítico consistiu em

pesar 2 g de solo em um tubo Falcon de 50 ml, no qual foram adicionados 40 ml de solução tampão de fosfato de sódio a pH 7,0 com diacetato de fluoresceína. Em seguida, o tubo foi fechado e incubado em rotação (120 rpm) a 35ºC por 24 horas. Após esse período, 2,0 ml de acetona foram adicionados para interromper a reação e o sobrenadante foi filtrado (Figura 2a) em papel filtro nº 42 (Química Moderna) para posterior leitura em um espectrofotômetro a 490 nm. Os mesmos procedimentos acima foram adotados para as réplicas de controle, exceto a adição do substrato (diacetato de fluoresceína).

Quantificação de urease

A análise da atividade da urease foi baseada na determinação de amônia liberada após incubação do solo com solução de ureia (TABATABAI; BREMMER, 1972). Cinco gramas de solo foram pesados e acondicionados em tubo falcon. Neste foram adicionados 2,0 ml de tolueno, 9,0 ml de tampão MUB com pH 9,0 e 1,0 ml de solução com ureia (0,2 M). Esta mistura foi mantida por 2 horas a 37ºC em uma câmara de incubação, em seguida 35 ml de cloreto de potássio e sulfato de prata (KCl 2M-Ag_2SO_4 100ppm) de solução aquosa foram adicionados para interromper a reação. Após agitação, a mistura foi deixada por cinco minutos à temperatura ambiente e o volume foi completado para 50 ml com solução de KCl-Ag_2SO_4, e agitado por alguns minutos. Pipetou-se 20 ml desta suspensão, na qual 0,2 grama de óxido de magnésio foram adicionados, e esta foi passada para um microdestilador. O destilado foi coletado em um Erlenmeyer contendo solução de ácido bórico e indicadores vermelho de metila e verdes de bromocresol (Figura 2b) que foram titulados com H_2SO_4 padronizado (0,005 M). O controle seguiu os mesmos procedimentos acima; no entanto, a ureia foi adicionada apenas após a solução KCl-Ag_2SO_4.

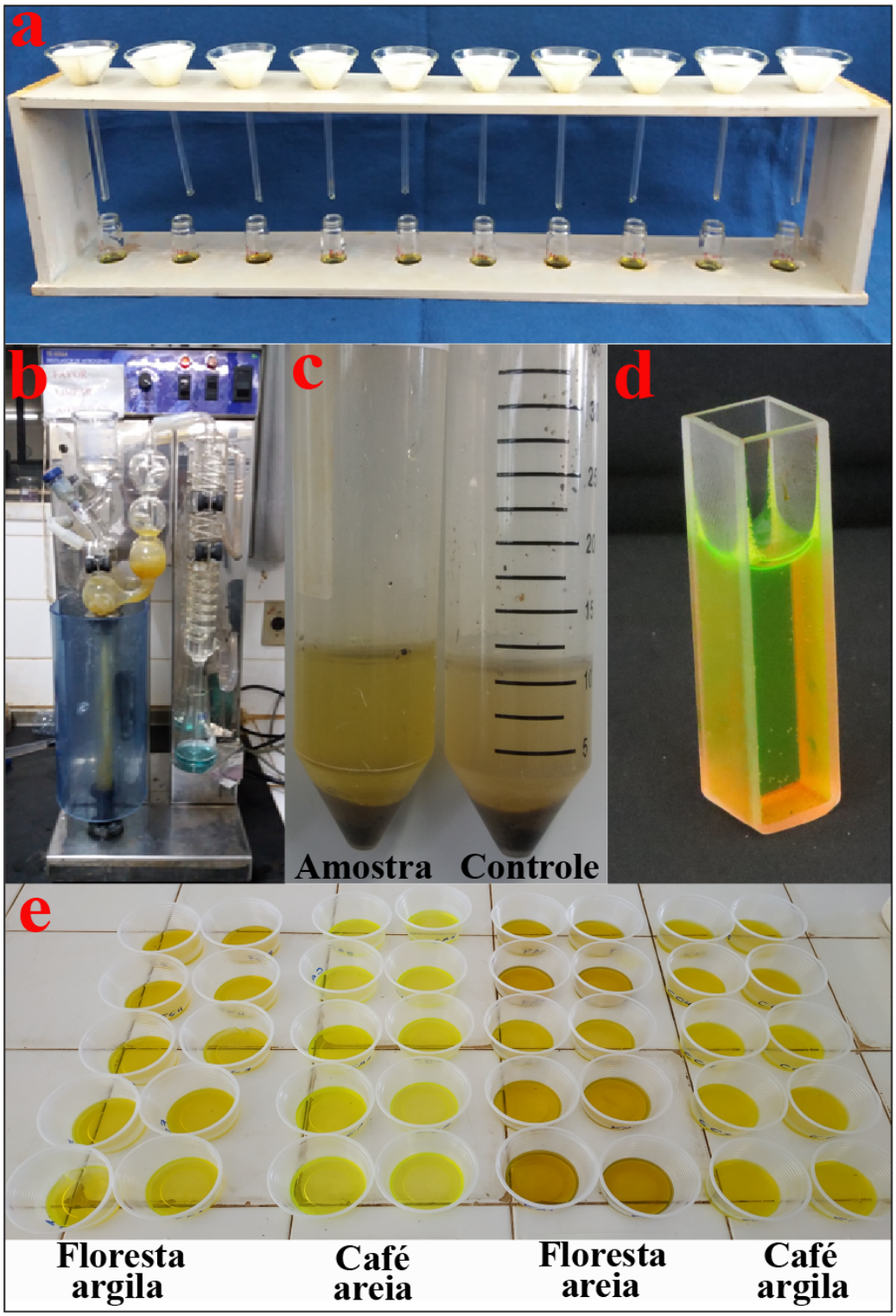
Quantificação da β-glucosidase, arilsulfatase e fosfatase ácida.

A análise da atividade da β-glucosidase foi baseada na determinação colorimétrica de ρ-nitrofenyl β-D-glucopyranoside (PNG) (DICK et al., 1996). Um grama de solo de cada amostra foi pesado, no qual foram adicionados 0,25 ml de tolueno, 1,0 ml de tampão universal modificado (MUB) a pH 6,0, e 1,0 ml de solução PNG (Figura 2c). Os tubos falcons com essa mistura foram agitados e incubados por 1 hora a 37ºC. Após esse período, foram adicionados 1,0 ml de $CaCl_2$ e 4,0 ml da solução tris hidroximetil aminometano (pH 12). O sobrenadante foi filtrado em papel filtro nº 42 e a cor amarelada foi lida em espectrofotômetro a 410 nm (Figura 2d).

A quantificação da arilsulfatase foi baseada no método descrito por Dick et al. (1996), que consiste no uso de Potassium ρ-nitrophenyl sulfate (PNS) como substrato. Um grama de solo foi pesado em um tubo Falcon, no qual foram adicionados 0,25 ml de tolueno, 4,0 ml de solução tampão acetato, e 1,0 ml de solução de PNS. Os tubos foram fechados e depois agitados por alguns segundos e incubados por 1 hora a 37ºC. Após esse período, a reação foi interrompida pela adição de 1,0 ml de $CaCl_2$ (0,5 M) e 4,0 ml de NaOH (0,5 M). Para o controle, foram adotados os mesmos procedimentos acima, exceto pela adição do substrato PNS, que foi colocado somente após a adição do $CaCl_2$ e do NaOH.

A análise da atividade da fosfatase ácida baseou-se na leitura em espectrofotômetro do ρ-nitrofenol (DICK et al., 1996). Em um grama de solo, foram adicionados 0,2 ml de tolueno, 4,0 ml de solução tampão universal (pH 6,5) e um ml de p-nitrophenyl disodium orthophophate (PNF) 0,05 M. A mistura foi incubada a 37 ºC por uma hora. Passado esse período, a reação foi interrompida pela adição de um 1,0 de $CaCl_2$ (0,5 M) e quatro ml NaOH (0,5 M) e agitada por alguns segundos. O controle foi formado com os mesmos procedimentos descritos acima, com exceção do PNF que foi adicionado somente após adição do $CaCl_2$ e NaOH. O filtrado dessas duas últimas enzimas (Figura 2e) também foi lido em espectrofotômetro a 410 nm.

Figura 2 – Ilustração dos procedimentos metodológicos de hidrólise do diacetato de fluoresceína e atividades das enzimas urease, β-glucosidade, fosfatase ácida e arilsulfatase. a) filtragem de amostras do solo, b) destilação da urease, c) amostras após incubação, d) cubeta para leitura em espectrofotômetro, e) amostras de enzimas após filtragem.



Fonte: Arquivos do projeto do autor

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SEGUNDA PARTE (Capítulo II e III)

ARTIGO 1

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The effectiveness of a microbiological attribute as a soil quality indicator depends on the storage time of the sample

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ABSTRACT

Purpose: The aim of this study was to analyze the effect of storage time on the main microbiological attributes used as soil quality indicators.

Methods: Seven attributes were evaluated (microbial biomass carbon, microbial basal respiration, fluorescein diacetate hydrolysis - FDA, and the activity of the enzymes urease, β-glucosidase, arylsulfatase, and acid phosphatase) in four different soil environments (clayey soil in forest, sandy soil in forest, clayey soil in coffee field, and sandy soil in coffee field). Each attribute was quantified at five different storage times (15 hours and 15, 30, 60, and 120 days).

Results: The values of FDA, microbial biomass carbon, and arylsulfatase declined significantly in the first 15 days of storage. Therefore, these attributes should be evaluated soon after soil collection. The values of microbial basal respiration declined on day 30; therefore, it should be evaluated up to day 15 of storage. The decline in urease values varied depending on soil texture; therefore, the shortest storage period, 15 days, should be considered. The values of β-glucosidase declined only on day 120. Therefore, soil samples can be stored for up to 60 days for determination of this enzyme. Acid phosphatase activity was not affected by storage time.

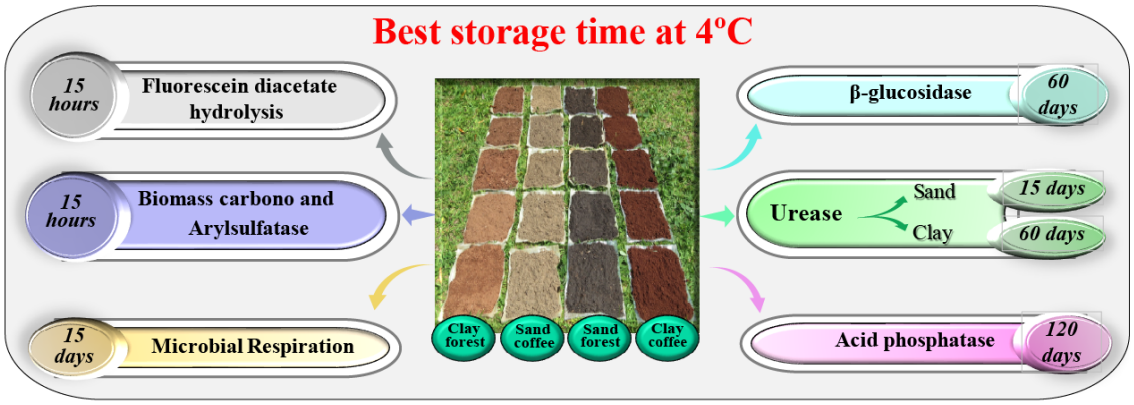
Conclusions: Depending on storage time, all the attributes were able to differentiate the soil environments. Long storage times compromise use of most of the attributes as soil quality indicators, as well as their usefulness in discriminating soil environments. Therefore, storage times should be considered when these attributes are used as soil quality indicators.

Keywords: Microbiological indicators, Enzymatic activity, Microbial biomass carbon, Microbial basal respiration, Soil storage.

Highlights

- The best storage time for soil samples at 4ºC is enzyme specific
- FDA, arylsulfatase, and MBC should be evaluated right after taking the soil sample
- Basal respiration and urease should be assessed within 15 days
- β-glucosidase should be evaluated within 60 days
- Acid phosphatase can be evaluated within at least 120 days

Graphical abstract



1 Introduction

One of the challenges of the twentieth century is implementation of agricultural systems that are able to ensure ecosystem sustainability. The development of effective tools is paramount for monitoring the health of these systems. Microbiological attributes are relevant in this context and should be integrated as an inseparable and harmonious element in soil studies. These attributes change significantly with small environmental variations, reflecting different levels of degradation. They are therefore directly related to soil quality (Nannipieri et al. 1990; Doran and Parkin 1994) and crop yield (Aragão et al. 2020).

The microbiological attributes most used to monitor soil quality are microbial biomass carbon, microbial basal respiration, and enzymatic activities (Dick et al. 1996; Doran and Parkin 1994). The enzymes most used for this purpose are related to the C, N, P, and S cycles (Gil-Sotres et al. 2005; Moreira and Siqueira 2006; Bastida et al. 2008). Nevertheless, the results presented in the literature regarding the usefulness of these attributes are often divergent, and this impedes comparisons among different studies. This divergence can be explained by characteristics intrinsic to each attribute and soil environment, but especially by the absence of methodological standards for these analyses. Gil-Sotres et al. (2005) carried out a meta-analysis of 1500 studies published on microbiological attributes of soil quality and reported that different manners of collection, pre-treatment of the samples, substrate concentration, and storage temperature and time are the main methodological reasons that give rise to contradictory results in studies. Storage is of decisive importance in these responses. Yet, the lack of detailed procedures in the methods of the articles evaluated shows the lack of rigor in this step, and few articles evaluate procedural aspects.

We reviewed 100 articles on the Web of Science and Scopus on the theme “microbiological indicators of soil quality” published in the last 15 years in periodicals with a high impact factor. Only 21 reported the storage time of the samples. That means that only 21% of the studies evaluated considered the importance of this variable. The samples were stored for up to 24 hours in 19% of the studies, for up to 14 days in 71%, for up to 30 days in 5%, and for more than 60 days in the other 5%. This temporal variability makes direct comparisons among these studies conducted in different laboratories impossible and may be one of the causes of incongruences among the results available in the literature.

Information regarding the effects of storage time on microbiological indicators of soil quality is insufficient and often contradictory. Tuner and Romero (2010) suggested that tropical soils can be stored for no longer than two weeks at either 4°C or 22ºC for evaluation of the β-glucosidase, acid phosphatase, and *N*-acetyl-β-D-glucosaminidase enzymes and of microbial phosphorus. DeForest (2009) did not observe clear and consistent patterns of the effect of storage time (21 days) at 4°C or -20ºC on the enzymatic activities of acidic forest soils. Wallenius et al. (2010) concluded that storage time is determined by the objective and experimental set up of the study; however, the author tested the effects of storage only on frozen and air-dried samples. These and other authors made conclusions concerning attributes in general, such as the need to store samples for as little time as possible (Ross 1965; Lee et al. 2007), but they considered that short storage time is not always possible (Burns et al. 2013) and, in some cases, they did not test different storage times (Alef and Nannipieri 1995; Bandick and Dick 1999). However, logistics, the distance of the sampling locations, the availability of human resources, and space limitations in laboratories often lead to the need for storage times well beyond the 24 hours or 14 days reported in the literature (ISO10381-6 2009; Turner and Romero 2010, respectively). Furthermore, the recommendation for a single storage time for all microbiological attributes does not take into account their specific characteristics and responses to storage, which compromises the validity of conclusions regarding the best indicators of soil quality under given conditions.

Lopes et al. (2015) recommended storage at room temperature, although they showed that β-glucosidase declined by 26% and arylsulfatase by 53% in tropical soils after two years of storage at room temperature. Many authors have also reported drastic reductions (> 50%) in enzymatic activity in temperate soils, and they did not recommend storing samples at room temperature (Ross 1965; Tabatabai and Bremner 1970; Pancholy and Rice 1972; Anderson 1987; Parham and Deng 2000; Lee et al. 2007; Wallenius et al. 2010). Although it has been noted that storage is enzyme specific, no useful practical information has been presented on adequate storage time (Wallenius et al. 2010). Additionally, as far as we know, there are gaps concerning the best storage time of samples for evaluation of several indicators, such as microbial biomass carbon, microbial basal respiration, hydrolysis of fluorescein diacetate, and urease and arylsulfatase activities, in tropical soils. Given this situation, we identified the need for a more specific study that considers the individual effect of storage on each attribute. Thus, the aim of this study is to provide scientists and laboratories with information needed to give priority

to those analyses that must be performed first, especially when many samples need to be handled. Thus, microbiological analyses can be performed with greater flexibility and more precise criteria.

Given this situation, we investigated the following hypotheses: Storage time at 4ºC affects the values of the attributes in tropical soils. Responses to storage time depend on the attribute and soil environments. Storage time affects the ability of the attribute to discriminate different soil environments. To test these hypotheses, soils with contrasting conditions and from different environments (sandy and clayey, from coffee fields and forests) were evaluated for the purpose of determining the effect of storage time on the main microbiological attributes used as indicators of soil quality. Specific objectives were (1) to determine how long the soil can be stored without compromising the effectiveness of the attributes in distinguishing different edaphic conditions; (2) to provide detailed information regarding storage; and (3) to provide an order of priority in analyses of soil attributes, based on sensitivity to storage time.

2 Methodology

2.1 Characterization of the areas and experimental design

The study was conducted with soil samples collected from environments under the same water regime (February 2019) in the municipalities of Lavras and Itumirim in the Alto Rio Grande region, belonging to the Atlantic Plateau geomorphological unit (Curi et al. 2017) in the South (region) of Minas Gerais in the southeast of Brazil. The geographic coordinates of the municipality of Lavras are 21°14’06” S, 45°00’00’’ W and altitude of 918 m AMSL, and of Itumirim, 21°15’57’ S, 44°50’49’’ W and 870 m AMSL. The climate in the region according to the Köppen classification is Cwa, temperate rainy (mesothermal), with a dry winter and wet summer and mean temperature of 20.4°C (Dantas et al. 2009).

The samples were obtained from four distinct soil environments. The first (clay-forest) is a native forest formed on an Oxisol, with clayey texture (Curi et al. 2017) and flat topography. The forest is a seasonal semideciduous mountain type (D’angelo Neto et al. 1998), with open canopy of around 15 meters height. The second (sand-forest) is a native forest formed on a Planosol, with a slightly accentuated slope in the direction of a stream. It is classified as a gallery forest (Loschi et al. 2013) and follows the water course, and the tree canopy at the two sides is around 10 m in height. The third (clay-coffee) is an experimental coffee (*Coffea arabica* L.) field, grown on an Oxisol of very clayey

texture and flat topography. The crop field does not have an irrigation system, and weed growth is controlled by mowing and depositing the plant biomass between the rows. The fourth soil environment (sand-coffee) is a specialty coffee (*Coffea arabica* L.) field, grown on a Planosol with a mild slope. The crop field is managed with application of NPK (10-28-20), has a drip irrigation system, and is mowed for weed control.

2.2 Soil collection and storage

For each microbiological attribute, the soil was sampled at the same storage time for each of the different soil environments (clay-forest, sand-forest, clay-coffee, and sand-coffee). Since these analyses are laborious and time consuming, each individual attribute was sampled at a different time (three days from analysis of one attribute to another). Thus, field samples were taken for microbial biomass carbon, urease, β-glucosidase, arylsulfatase, fluorescein diacetate hydrolysis, microbial basal respiration, and acid phosphatase on February 4, 7, 10, 13, 16, 19, and 22 of 2019, respectively. The climatic conditions of this period are shown in Fig. 1. The sampling procedure consisted of collection of five compound samples (n = 5), equally spaced (50 m) in a 250 m transect in each soil environment. Each sample was composed by mixing four subsamples obtained at a depth of 0-10 cm. In the forests, the plant litter was carefully removed, and single samples were taken in a circle around the sampling point with the aid of a shovel. In the coffee-growing areas, soil samples were taken in a circle under the plant canopy. Samples with the same moisture content from the field were sieved in 2-mm mesh screens, and a 400-g aliquot was removed for physical and chemical analyses. For the microbiological analyses, the soil was placed in plastic bags and then in cold storage at 4ºC. Storage at 4ºC is recommended and widely used in microbiological trials since this temperature minimizes the effect of enzyme inactivation in short storage periods (Ross 1965; ISO 1993; Lee et al. 2007; DeForest 2009; Abellan et al. 2011). Each microbiological attribute was measured at five different storage times (15 hours and 15, 30, 60, and 120 days). The time of 15 hours after collection for the first measurement was adopted because analyses performed immediately after collection can overestimate the values of the attributes (Petersen and Klug, 1994, Turner and Romero, 2010). This may occur due to release of intracellular enzymes from roots and fungal hyphae because of disturbing the soil during sampling and processing, resulting in an increase in microbiological activity (Petersen and Klug, 1994).

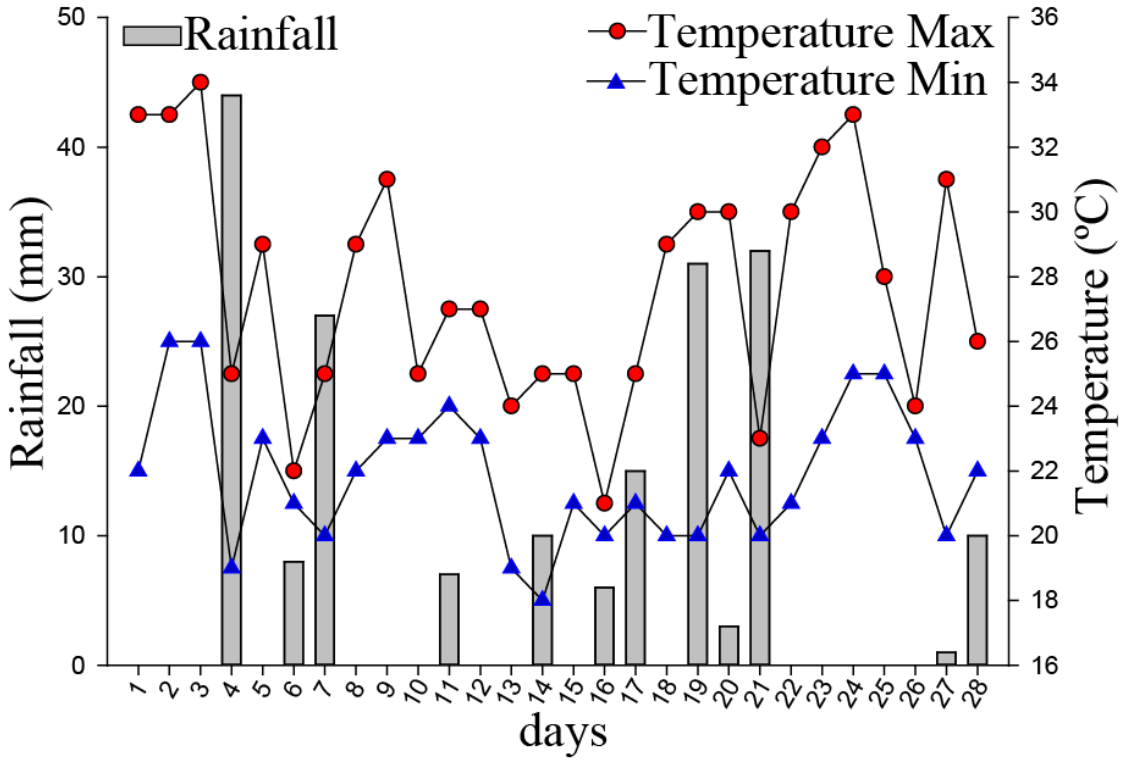


Fig. 1 Climatic conditions in the collection period of soil samples for microbiological analyses (February 2019).

2.3 Physical and chemical analyses

The physical and chemical attributes evaluated were pH, measured in a soil and water suspension (1:2.5); phosphorus (P), potassium (K), zinc (Zn), manganese (Mn), and copper (Cu), extracted by the Mehlich-1 solution (Mehlich 1953); calcium (Ca), magnesium (Mg), and aluminum (Al), extracted by 1 mol L^{-1} KCl (McLean et al. 1958); potential acidity (H + Al) extracted by the SMP extractant (Shoemaker et al. 1961); sum of exchangeable bases (SB); base saturation (V); aluminum saturation (m); cation exchange capacity at pH 7.0 (T); sulfur (S), extracted by monocalcium phosphate in acetic acid (Hoeft et al. 1973); organic matter (MO), by oxidation with potassium dichromate in acid medium (Walkley and Black 1934); and texture, by the Bouyoucos method (Bouyoucos 1951). Physical and chemical characterization of the locations studied is shown in Table 1.

Table 1 Chemical and physical attributes of soil environments studied.

soil environment	Chemical attributes								
	pH	P	K	Ca^{2+}	Mg^{2+}	S	Mn	Cu	Zn^{+2}
		------mg dm^{-3}-------		-----$cmol_c$ dm^{-3}-------		----------mg dm^{-3}----------			
Clay-forest	4.5 † ± 0.3††	4.2 ± 0.7	75 ± 7	3.2 ± 0.8	0.8 ± 0.2	4.7 ± 1.7	70.6 ± 23	2.9 ± 0.3	2 ± 0.4
Sand-forest	4.6 ± 0.3	11 ± 1.4	133 ± 60	1.4 ± 0.9	0.6 ± 0.3	3.3 ± 0.3	14.3 ± 8.8	0.8 ± 0.4	3 ± 0.4
Clay-coffee	4.8 ± 0.3	134 ± 32	583 ± 42	3.5 ± 0.2	0.7 ± 0	13 ± 3.1	12.0 ± 1.2	5.6 ± 0.4	16 ± 2
Sand-coffee	5.5 ± 0.1	351 ± 89	147 ± 46	1.5 ± 0.1	1.2 ± 0.1	1.4 ± 0.3	15.0 ± 2.3	6.3 ± 1.2	41 ± 34

soil environment	Chemical attributes						Physical attributes		
	SB	H+Al	T	V	m	OM	Clay	Silt	Sand
	----------$cmol_c$ dm^{-3}----------			---------%--------		$dag\ kg^{-1}$	---------- $dag\ kg^{-1}$----------		
Clay-forest	4.3 ± 1.0	10 ± 2.3	14 ± 3.2	29 ± 2	10 ± 3.3	7.3 ± 1.3	35 ± 4	20 ± 2	44 ± 6
Sand-forest	2.4 ± 1.1	17 ± 3.2	19 ± 3.1	12 ± 5	41 ± 17	6.4 ± 1.0	18 ± 3	16 ± 6	64 ± 5
Clay-coffee	5.7 ± 0.5	8.9 ± 2.1	14 ± 2.0	39 ± 7	2.7 ± 0.8	4.5 ± 0.5	49 ± 1	23 ± 1	27 ± 2
Sand-coffee	3.1 ± 0.3	3.3 ± 0.3	6 ± 0.4	48 ± 3	3.1 ± 0.3	2.0 ± 0.1	15 ± 1	6.4 ± 1	78 ± 1

†Means obtained from a value n = 5, ††Standard deviation. SB: sum of exchangeable bases, H + Al: potential acidity, T: cation exchange capacity at pH 7.0; V: base saturation, m: aluminum saturation, OM: organic matter.

2.4 Microbiological analyses

The following microbiological attributes were evaluated: microbial biomass carbon, microbial basal respiration, fluorescein diacetate hydrolysis (FDA), and activities of the enzymes urease (EC 3.5.1.5), β-glucosidase (EC 3.2.1.21), arylsulfatase (EC 3.1.6.1), and acid phosphatase (EC 3.1.3.2).

The microbial biomass carbon was determined by the fumigation and extraction method (Vance et al. 1987). The estimate of evolved CO_2 - microbial basal respiration during the incubation process followed the protocol of Jenkinson and Powlson (1976).

Quantification of FDA by microbial cells was determined by fluorescein release, using fluorescein diacetate lipase as a substrate, according to Dick (1996). Urease activity was based on determination of ammonia released after incubation of the soil with urea solution (Tabatabai and Bremmer 1972).

The enzymes β-glucosidase, arylsulfatase, and acid phosphatase were quantified by colorimetric determination of ρ-nitrophenol using 4-nitrophenyl β-D-glucopyranoside, potassium 4-nitrophenyl sulfate, and 4-nitrophenyl disodium orthophosphate as substrates, respectively (Dick et al. 1996). Modifications in the above methods are described in Aragão et al. (2020). The same brands of reagents were used in all the analyses and enzymatic substrates. For enzymatic analyses, lower cost filters that were

used were compared to the filters of the original method (Whatman) to ensure consistent results.

2.5 Data analysis

Analysis of variance was performed on the data, and after validation of the statistical model, a multiple comparison was made based on mean cluster analysis by the Scott-Knott algorithm at the 0.05 significance level in the programming language R 3.3.1. After that, the values were used for regression analysis and models with the best fit were chosen with the assistance of the Curve Expert Professional 2.6.5 program. The figures were made with Sigma Plot 12.5 and Illustrator CC 2017 21.0.0.

3 Results

All the attributes were able to discriminate the different soil environments evaluated; however, this ability declined with the increase in storage time (Fig. 2, 3, 4a, and 4b). Storage time significantly affected the values of microbial biomass carbon, microbial basal respiration, diacetate hydrolysis, and activity of the enzymes β-glucosidase, urease, and arylsulfatase, but it did not affect acid phosphatase activity (Fig. 4c).

3.1 Microbial biomass carbon and microbial basal respiration

The effect of storage time on microbial biomass carbon did not depend on soil texture or land use (Fig. 2a). For the clay-forest, the linear model best fit the data, showing a constant decrease in microbial biomass carbon over storage time. The sand-forest and clay-coffee soil environments were described by the logistic power and quadratic models, respectively (Fig. 2a). According to the models, the microbial biomass carbon declined in the first few days and the rate of decline decreased over prolonged storage times. This response is best observed in the sand-forest and clay-coffee soil environments. A reduction of 14% in sand-forest and 26% in clay-coffee was observed between 15 h and day 15 (Fig. S1a). The logistic power model (Fig. 2a) best described the decline in microbial biomass carbon in the sand-coffee soil environment. The response of this variable was stable up to day 30 of storage, as observed in mean cluster analysis, which showed statistically significant differences beginning only at day 30. The microbial biomass carbon decreased 46% from day 30 to 60 and 37% from day 60 to 120.

The measurements of microbial basal respiration in all sample types were affected by storage time (Fig. 2b). The most accentuated effect was observed in samples from the clay-forest and sand-forest environments, which exhibited an average reduction of 87% from 15 hours after sampling to 120 days of storage. The microbial basal respiration in these soil environments was described by a logistic power model with coefficient of determination (R^2) of 0.80 for clay-forest and a reciprocal quadratic model with R^2 of 0.86 for sand-forest (Fig. 2b). The reduction from 15 hours after sampling to day 15 was 19% for the clay-forest samples and 14% for the sand-forest samples. However, this reduction was considered to be statistically insignificant by complementary mean cluster analysis (Fig. 2b) ($p > 0.05$). Significant reductions of 56% for clay-forest and 46% for sand-forest samples up to day 30 were observed. The decline in microbial basal respiration was best described by logistic power and quadratic models for samples from the clay-coffee and sand-coffee sites, respectively (Fig. 2b). These models were able to describe 90% (clay-coffee) and 85% (sand-coffee) of the total variability of microbial basal respiration. According to the multiple comparison test, the reduction in microbial basal respiration in samples from both soil environments was not significant up to day 30. The differences became significant only after this period (Fig. S1b).

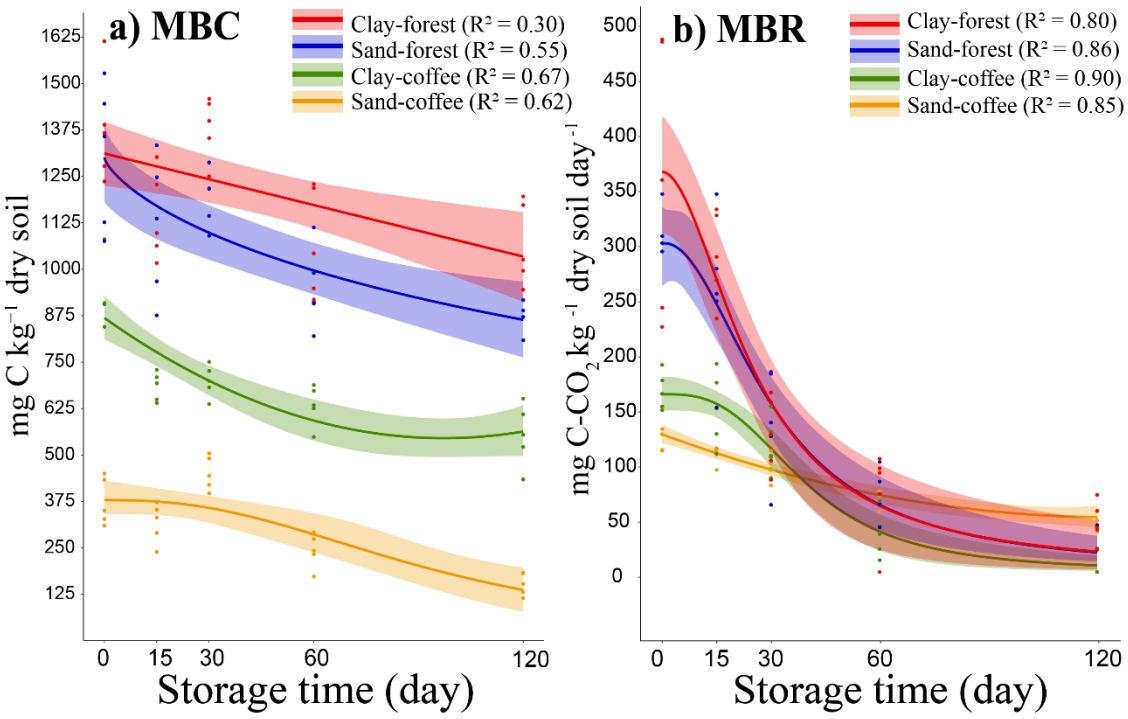


Fig. 2 Variation in storage time in a) microbial biomass carbon (MBC) and b) microbial basal respiration (MBR) in soil samples from sandy and clayey soils under forest and

coffee-growing environments. Zero time corresponds to the evaluation carried out 15 hours after soil collection. Equations of lines for MBC: clay-forest (y=-2.31**x+1311.6***); sand-forest (y = 1300.65***/(1+(x/306.5*)0.728*)); clay-coffee (y = 0.034**x^2 - 6.66***x+868.82***); and sand-coffee (y = 378.7***/1 + (x/95.1***)2.45*). ***). Equations of the lines for MBR: clay-forest (y=369.4***/(1+(x/25.52***)1.89***)); sand-forest (y=1/(0.003***-0.000005x+0.000003*x2)); clay-coffee (y=164.19***/(1+(x/39.6***)2.95***); and sand-coffee (y = 0.005**x^2-1.22***x+127.13***). *Significant at the 0.1 probability level, **significant at the 0.05 probability level, and ***significant at the 0.01 probability level.

3.2 Fluorescein diacetate hydrolysis (FDA) and urease activity

Among the attributes evaluated, FDA was most affected by storage time, regardless of soil texture and land use. All combinations of soil texture and soil environments were adequately described by logistic models (Fig. 3a). The models represented 98%, 96%, 95%, and 86% of the total variability of the data for clay-forest, sand-forest, clay-coffee, and sand-coffee, respectively. They describe an extreme reduction in FDA in the first few days of storage. The rate of decline decreases over time and stabilizes at day 30. Although the responses of this attribute varied in the same direction, the magnitudes differed according to sample type. From 15 hours to day 15, FDA declined 65, 51, 41, and 23 % in the samples from the clay-forest, sand-forest, clay-coffee, and sand-coffee environments, respectively (Fig. S1c).

The effect of storage time on urease activity was more dependent on soil texture. Urease activity was more sensitive to storage time in samples from sandy soils; however, the activity in these soils declined only after day 15 (Fig. 3b). Urease activity declined 13% from 15 day to day 30 of storage in the sand-forest samples; the decline continued gradually, with the greatest reduction (50%) observed from days 60 to 120. For the sand-coffee samples, activity decreased moderately and stabilized from days 60 to 120. For the clayey soils, the models describe a stable response of the enzyme up to day 60 of storage. However, after this period, there was an extreme change in activity, with a reduction of 68% in clay-forest and 50% in clay-coffee samples from day 60 to day 120 (Fig. S1d).

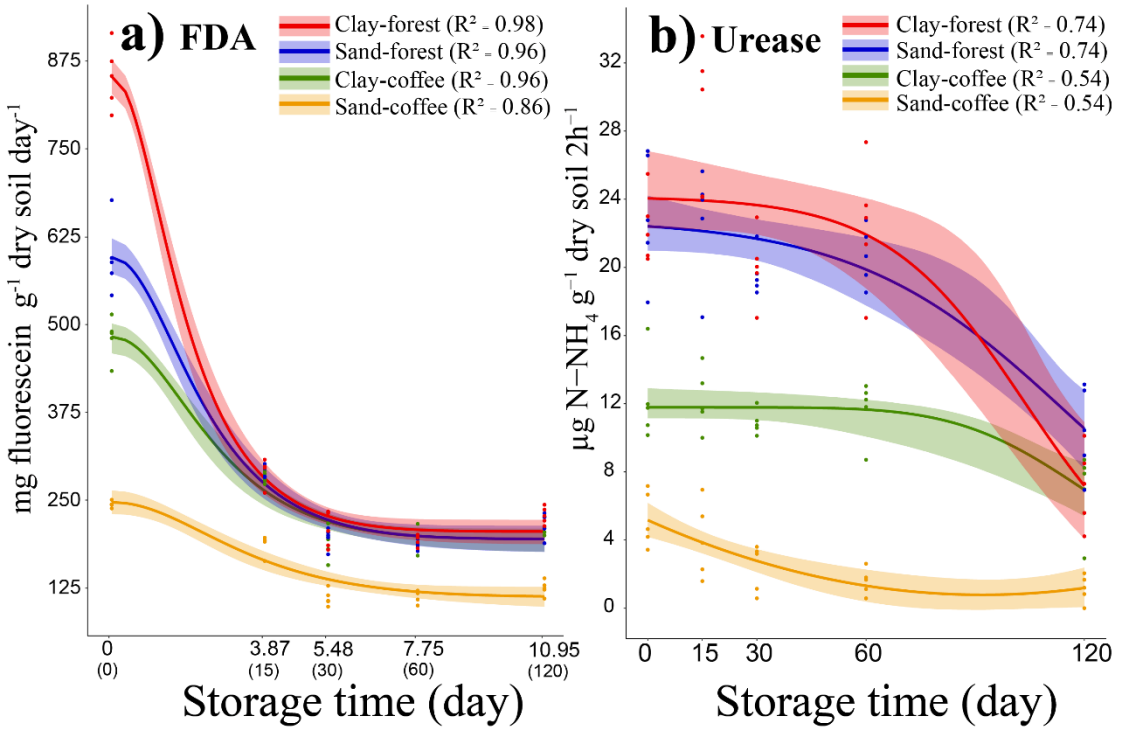


Fig. 3 Variation in storage time in a) fluorescein diacetate hydrolysis (FDA) and b) urease activity in soil samples from sandy and clayey soils under forest and coffee-growing environments. Zero time corresponds to the evaluation carried out 15 hours after soil collection. Equations of lines for FDA: clay-forest (y = 204.79***/(1-0.75****exp(-0.069***x))); sand-forest (y = 194.06***/(1-0.67**** exp(-0.056***x))); clay-coffee (y = 193.99***/(1-0.6****exp(-0.055***x))); and sand-coffee (y = 111.74***/(1-0.54**** exp(-0.036**x))). Equations of lines for urease: clay-forest (y = 24.16***/(1+exp(-5.42*+(0.052x*)))); sand-forest (y = 22.16***/(1+exp(-3.97*+(0. 033x*))); clay-coffee (y = 11.79***/(1+(x/127.52***)5.96)); and sand-coffee (y = 0.0005x²*-0.0952x**+5.169***). *Significant at the 0.1 probability level, **significant at the 0.05 probability level, and ***significant at the 0.01 probability level.

3.3 β-glucosidase, arylsulfatase and acid phosphatase activity

In the first few days, β-glucosidase activity was little affected by storage time. For clay-forest, sand-forest, and sand-coffee samples, reduction in the activity of this enzyme was not significant up to day 60 (Fig. 4a). After this period, β-glucosidase activity in samples from all soil environments differed significantly, with reduction of 47%, 38%, 52%, and 53% on day 120 for clay-forest, sand-forest, clay-coffee, and sand-coffee, respectively (Fig. S1e).

Arylsulfatase activity decreased with increased storage time; however, the responses of activity of this enzyme varied with the land use and soil texture combinations, showing no consistent pattern of decline. The quadratic model for clay-forest and logistic power model for sand-coffee represented the total variability of the data, with R^2 of 0.80 for clay-forest and 0.86 for sand-coffee. These models showed an extreme reduction in enzymatic activity up to day 30; after that period, the reduction was moderate (Fig. 4b). The reduction from 15 hours after storage to day 15 was 44% for clay-forest and 53% for the sand-coffee soil samples. The models described the total variability of the data, with R^2 of 0.81 for sand-forest and 0.79 for clay-coffee. In these soil environments, activity remained constant up to day 15 of storage. The activity declined after that period, exhibiting a 47% reduction for sand-forest and 73% reduction for clay-coffee from day 15 to day 30 (Fig. S1f).

Storage time did not affect the activity of acid phosphatase in any of the soil environments evaluated (Fig. 4c and Fig. S1g).

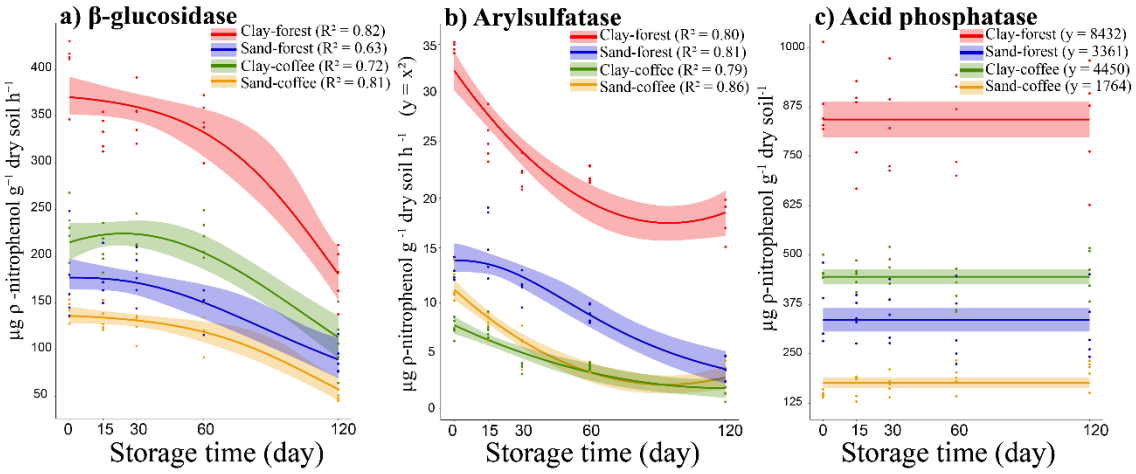


Fig. 4 Variation in storage time in a) β-glucosidase, b) arylsulfatase, and c) acid phosphatase activity in soil samples from sandy and clayey soils under forest and coffee-growing environments. Zero time corresponds to the evaluation carried out 15 hours after soil collection. Equations of lines for β-glucosidase: clay-forest (y = 3766.37***/(1+exp(-4.14**+(0.035**x)))); sand-forest (y = 1774.03***/ (1+ (x/121.16***)2.46*)); clay coffee (y = 2246.26****exp(-(x-24.07*)²/(2*82.05²***))); and sand-coffee (y = 1392.13 ***/(1+exp(-3.91**+(0.035**x)))). Equations of lines for arylsulfatase: clay-forest (y = 0.005***x²-0.95***x+101.46***); sand-forest (y = 44.53***/(1+(x/76.51***) 2.24***)); clay-coffee (y = 0.0013*x²-0.31***x+25.07***); and sand-coffee (y = 0.003***x²-0.61***x+35.77***). For acid phosphatase y = mean. *Significant at the 0.1

probability level, **significant at the 0.05 probability level, and ***significant at the 0.01 probability level.

4 Discussion

In order to establish suitable storage times, we chose contrasting soil environments (with and without plant diversity) and contrasting soil textures (sandy and clayey). These factors can affect microorganism survival (Zantua and Bremner 1977; Burns 1982; Flores-Rentería et al. 2020) and thus affect the degree of microbial sensitivity to storage. In this study, the microbial communities were more sensitive to storage in the soil samples coming from the clay-forest, sand-forest, and clay-coffee soil environments (Fig. 2a). This sensitivity may be associated with the carbon supply in these environments (Table 1). The mean microbial biomass carbon values at 15 hours ranged from 930 mg C kg^{-1} of dry soil in the clay-coffee sample to 1377 mg C kg^{-1} in the forest environment sample (Table 2), which indicates high microbial biomass in these environments (Lopes et al. 2013). Reduction in microbial biomass over storage time may be associated with an increase in decomposition of easily oxidizable organic compounds (Coxson and Parkinson 1987). This increase is caused by greater availability of oxygen brought about by soil turnover at the time of sample collection (Burns et al. 2013). Without replacement of energy sources, the community will decrease until reaching stability. This process was adequately shown in Fig. 2b, in which a significant reduction in microbial biomass carbon was observed in the first few days of storage, but then values did not change from day 60 to day 120.

The reduction in microbial biomass carbon, followed by an increase from day 15 to day 30 (Fig. 2b), may be related to the priming effect on the microbial community of the clay-forest environment (Hamer and Marschner 2005). This may have arisen from microbial competition for energy sources available during soil storage, which may have led to the death of some groups and promotion of others, such as r-strategists, increasing their biomass (Fontaine et al. 2003). Similar to the response in other soil environments, the community declines once more because of limitation of energy, with only k-strategist microorganisms remaining, which are continually active since they use more recalcitrant organic compounds (Kendrick and Burges 1962). The predominance of this latter group of microorganisms in the soil explains the stability of microbial biomass carbon from day 60 to day 120 of storage.

Storage time had a different effect on microbial biomass carbon in the soil environments evaluated, and reduction occurred in the first 15 days. This leads to the recommendation that this attribute be evaluated as quickly as possible. Otherwise, microbial biomass carbon will be underestimated and its magnitude in the hierarchy of the soil environments will be reduced.

The different models that describe microbial basal respiration in the four soil environments show that microbial activity was greatly affected by storage time. Such results are consistent with those observed in microbial biomass carbon, in which there was also a reduction from the first few days on. The greatest reductions in microbial basal respiration occurred where it was originally greater (clay-forest and sand-forest). This may be due to interruption of the addition of organic matter caused by removal of the soil sample from its natural environment (Hamer and Marschner 2005). The effect of storage conditions on microbial oxidative capacity is added to this. In general, the rates of microbial reactions are higher at temperatures of around 28ºC and decrease below 25ºC (Moreira and Siqueira 2006). Considering that the soil was stored at a temperature of 4ºC, this condition may have interfered in microbial activity. However, the limited availability of studies does not allow a more precise conclusion regarding the effect of storage temperature on this attribute. Thus, it is essential that storage time not exceed 15 days, because there is a sharp reduction in the values of this attribute even after short storage times (Fig. 2b). Nevertheless, under limiting conditions and in an exceptional manner, samples can be conserved for a maximum of 30 days when taken from agricultural ecosystems.

Among the attributes evaluated, FDA was most affected by storage time, and a transformation on the x axis ($x = y^2$) was necessary for better visualization of the overlapping of the models regarding the different soil environments. Overlapping shows that an increase in storage time leads to loss of capacity to differentiate the soil environments (Fig. 3a). In forest soils, the FDA was most affected by storage time. This reduction is directly related to reduction in viable cells from the soil since FDA is strongly correlated with microbial basal respiration (Schnurer and Rosswall 1982). In fact, the shape of the curves in the regressions of these two attributes shows that they respond in a similar way to storage (Fig. 2a and 3a), i.e., dormancy and death of microbial cells affected FDA. The fluctuation of microbial communities in the soil may also have contributed to reduction in FDA (Fig. 2a). As fluorescein diacetate is hydrolyzed by enzymes (proteases, lipases, and esterases) mainly produced by fungi and bacteria

(Guilbault and Kramer 1964; Schnurer and Rosswall 1982), the factors that affect these microorganisms affect the production of enzymes and, consequently, affect FDA. In addition, these enzymes are mainly extracellular, i.e., they are linked to external membranes of the cell or are excreted to the environment. Thus, they become more vulnerable to biodegradation (hydrolysis by other enzymes) (Burns, 1986). Another aspect that may be associated with reduction in the values of FDA is exposure of the samples to long periods of low temperature (4ºC). That may have led to enzymatic inactivation over time, since the greatest efficiency of FDA occurs at temperatures of 35ºC (Diack 1997; Jia et al. 2015). Given the limited availability of studies on the influence of temperature on this attribute, we recommend that quantification be performed with the soil still fresh, immediately after collection. That way, the differences expected among soil environments are more likely to be observed (Fig. 3a). In the event of limitations, the soil can be stored, however, for a period of less than 15 days.

Urease activity was not affected by storage time from 15 hours to day 15. This result may be due to the type of interaction of this enzyme with the soil constituents and to their capacity of protecting it, for a certain period, from biodegradation (Zantua and Bremner 1977). Unlike the FDA enzymes, urease is mentioned in the literature (Burns 1982; Burns 1986; Moreira and Siqueira 2006) as an enzyme with high hydrolytic efficiency in the soil structural microunits with a diameter of less than 50 µm. These reports support the results of this study, especially in the samples from the clay-forest and clay-coffee soil environments, which proved to be more resistant to storage time (Fig. 3b). In the soils from these environments, there is predominance of organo-mineral complexes by the association between organic molecules and silicate clays and/or iron and aluminum oxides and hydroxides (Schulten and Leinweber 2000; Christensen 2000). This association forms the “microbial microenvironments” that can protect urease (Burns 1986). These results are consistent with the findings of Pancholy and Rice (1972), who did not observe a reduction in activity in samples stored at 4ºC for 30 days. However, as observed in this study, this enzyme may be subject to loss of activity as time passes as catalytic sites will become less available because of diffusion of urea in the soil. An additional explanation is the effect of storage on the microbial community (Fig. 2a). As urease is mainly produced by microorganisms of the rhizosphere (Estermanm and McLarem 1961), their reduction may lead to a decrease in the abundance and activity of this enzyme. This process was clearly observed from day 15 to day 30 for sandy soils, and from day 60 to day 120 for clayey soils (Fig. 3b). Thus, it is recommended that

quantification of this enzyme in sandy soil samples be performed within the first 15 days after collection. For samples coming from clayey soils in agricultural soil environments, however, maximum storage time at 4ºC can be up to 60 days. Thus, considering that urease responses depend on the type of soil sample, it is better for evaluations to be made within the first 15 days after sample collection.

Resistance of β-glucosidase to storage up to day 60 in the soil environments studied may be associated with the abundance of these enzymes in the soil (Bhatia et al. 2002). This characteristic allows greater ability to obtain energy by cellulose degradation. This process mainly occurs through selective cleavage of the β-1,4-glycosidic bond in various disaccharides, oligosaccharides, glycoconjugates, aminoglycosides, and alkyl- and aryl-β-d-glycosides (Bhatia et al. 2002; Yang et al. 2008). Since cellulose is a very abundant biopolymer in the environment (Yang et al. 2008), these enzymes may have been favored by such an energy source, remaining active for a longer time in the soil (Turner and Romero 2010). However, during storage, there was no replacement of this energy source, which may have affected activity from day 60 of storage on (Fig. 4a). In addition, these enzymes are extracellular (Parham and Deng 2000) and may be biodegradable beginning at a certain period (60 days in the case of this study). Therefore, for β-glucosidase activity, reliable results may be obtained up to day 60 of storage.

The sensitivity of the arylsulfatase enzyme to storage time may be associated with the form in which this enzyme occurs in the soil. Arylsulfatases form an important group of enzymes that catalyze the hydrolysis of esters of organic arylsulfates (Tabatabai et al. 1994). A considerable part of these enzymes are found outside of the cells and are mainly secreted by bacteria (McGill Colle 1981). In the environment, they may be immediately metabolized by microorganisms or form protein-colloid complexes (Burns 1982; Moreira and Siqueira 2006; Balota et al. 2013). Interaction with the physical, chemical, and biological medium may result in adsorption, denaturation, and biodegradation of this enzyme, reducing its activity over time (Burns 1982; Moreira and Siqueira 2006). In addition, the factors that affect the soil microbial communities can affect the activity of this enzyme, since it is mainly produced by microorganisms (McGill Colle 1981). Tabatabai and Bremner (1970) observed a reduction of 18% in 3 months and Lopes et al. (2015) reported a 53% reduction in arylsulfatase activity in 2 years in air-dried samples stored at room temperature. These authors attributed the reduction in activity to the effect of air drying on the viable microbial community of the soil. Our study did not show a consistent pattern of the effect of soil texture and type of land use

on arylsulfatase activity, making it necessary to quantify it as quickly as possible for all soil environments.

The lack of effect of storage time on acid phosphatase activity may be a result of the mechanisms of cell protection for this enzyme. When such enzymes are secreted, they remain in the external cortical cells of the roots and especially in the plasmalemma (Ladd 1978). The plasmalemma, with its large quantity of phospholipids, has the function of coating, protecting, and creating the selective permeability of the cell. Thus, the acid phosphatases may have been benefitted by these mechanisms, as they are more stable in the soil. As a consequence, this may have favored maintenance of the molecular structure of acid phosphatase. Likewise, DeForest (2009) did not observe a reduction in acid phosphatase activity even after 21 days when acidic soil was stored at 4ºC. Nevertheless, Lopes et al. (2015) observed a reduction of 72% in the activity of this enzyme in air-dried samples stored for two years at room temperature, which may have been a consequence of enzyme denaturation. Our results corroborate that storage at 4ºC preserves acid phosphatase activity in tropical soils for long periods, thus providing greater flexibility of storage time when a large number of samples must be processed.

The results of this study show that the effect of storage time depends on each attribute. In short, the variable responses may be related to the way in which each enzyme is associated with the soil matrix. The greater affinity of phophatase and urease with the smaller fractions of the soil (Burns 1986; Marx et al. 2005) may have contributed to the greater stability of these enzymes over time. In addition, the size of the fractions affects the diffusion of the substrate (Handriková et al. 1996) and may explain the variable catalytic efficiency among the enzymes (Marx et al. 2005; Bailey et al. 2012). Substrate availability is fundamental for enzyme viability and activity in the soil and may be related to maintenance of the β-glucosidase activity that degrades cellulose, a compound that is very abundant in the soil (Yang et al. 2008). Enzymatic dependency on live cells also contributes to an explanation. Such is the case of the enzymes that degrade fluorescein diacetate (Schnurer and Rosswall 1982). In addition, storage can affect soil pH (Turner and Romero 2009), which, associated with temperature and humidity, affect cell stability (Baldrian et al. 2012; Zhang et al. 2018), causing reduction in FDA and in microbial biomass carbon. Microbial basal respiration is also affected by the quantity of active cells; however, other disturbances of the environment can make a small unit of microbial biomass have high respiration (Trujillo-Narcía et. 2019). The pattern of the effect of storage time on arylsulfatase activity is less clear, possibly through its relation to

microbial biomass, the main source of production of this enzyme de (McGill Colle 1981). However, arylsulfatase can form protein complexes with soil mineral fractions (Burns 1982), which may explain the temporary stability in two of the soils studied (clay-coffee and sand-forest). Additional studies, however, may be useful in better clarifying the variable response patters of these attributes, for example, monitoring the effect of storage on the kinetic enzyme system. Enzyme activity does not depend only on the amount of enzymes, but also on more active enzyme systems (Marx et al. 2005).

Arriving at consensus on storage time of samples is fundamental. Differences in this respect may compromise the effectiveness of these attributes in discriminating different soil environments and may be one of the causes of the incongruence observed among the results of diverse studies regarding this subject. For that reason, it is crucial to standardize the experimental conditions and procedures, including soil sample storage times and conditions, because this is a fundamental factor in interpretation of values of the attributes (DeForest 2009). In other words, the scientific community needs to make a coordinated effort in the sense of faithfully representing the conditions of the different soil environments. This may be possible through knowledge of the soil storage time suitable for evaluation of each attribute. The aim of our study was to make a contribution towards reaching this goal. Our findings are relevant for guiding soil quality studies in the context of tropical soils.

5 Conclusions

The storage time of soil environments used for quantification of microbiological attributes significantly affects the data that are generated and can change the way these data are interpreted, leading to erroneous conclusions and compromising the use of these microbiological attributes as soil quality indicators. Therefore, the results of this study lead to the following conclusions: (1) The best storage time for soil samples at 4ºC is enzyme specific; (2) FDA, arylsulfatase, and microbial biomass carbon should be evaluated right after taking the soil sample; (3) Basal respiration and urease should be assessed within 15 days; (4) β-glucosidase should be evaluated within 60 days; (5) Acid phosphatase can be evaluated within at least 120 days.

Thus, the following order of priority is recommended for microbiological analyses in accordance with their sensitivity to storage: 1st - fluorescein diacetate hydrolysis / microbial biomass carbon / arylsulfatase; 2nd - microbial basal respiration / urease; 3rd - β-glucosidase; and 4th - acid phosphatase.

Adoption of this order, taking into consideration the maximum storage limit of each attribute, may resolve possible incongruences among different studies and increase the effectiveness of the results, allowing greater advances in studies regarding soil quality.

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Credit authorship contribution statement:

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André Alves Souza: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing.

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Supplementary material

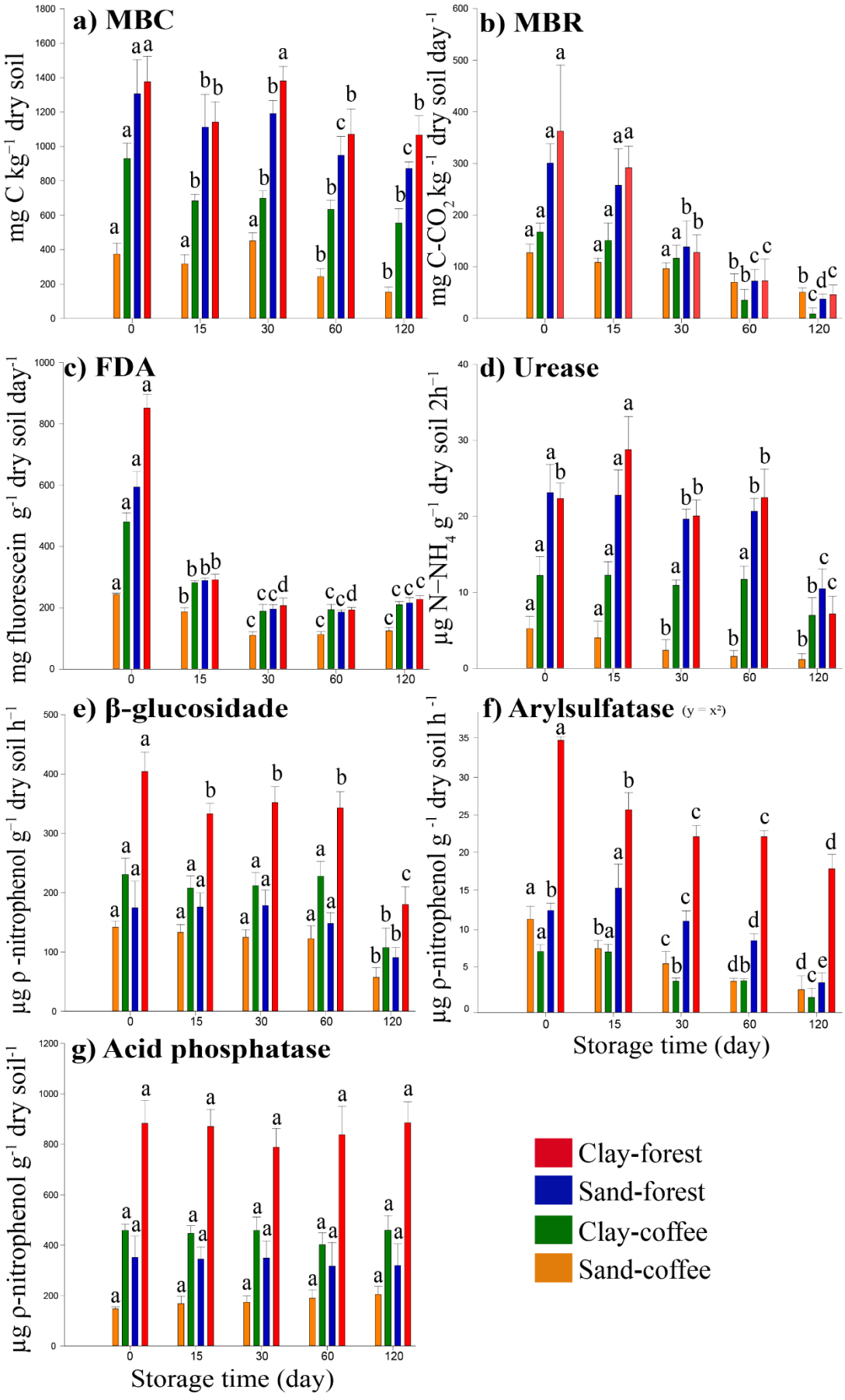

Fig. S1 Variation in storage time in a) microbial biomass carbon (MBC), b) microbial basal respiration (MBR), c) fluorescein diacetate hydrolysis (FDA), d) urease, e) β-glucosidase, f) arylsulfatase, and g) acid phosphatase activity. Zero time corresponds to the evaluation carried out 15 hours after soil collection. The letters compare the different days of storage for each soil environment studied at the 0.05 probability level (Scott-Knott). Bars represent means of five replications (n = 5).

ARTIGO 2

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Microbiological attributes as soil quality indicators for coffee fields and adjacent forests in the Brazilian Atlantic Forest biome

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ABSTRACT- Ensuring soil quality of coffee fields is fundamental for sustainable production of coffee itself. Microbiological attributes are especially effective predictors of changes in the soil. But their value as indicators can vary depending on the soil class and plant cover. This study aimed to determine the effect of different soil classes and plant covers on microbiological attributes and the potential of these attributes as indicators of the quality of different soils used for conventional coffee growing in the Atlantic Forest domain. Two coffee fields and two adjacent forests formed on *Oxisol* and *Planosol* were studied. The microbiological attributes evaluated were microbial biomass carbon - MBC, microbial basal respiration - MBR, fluorescein diacetate hydrolysis,

metabolic quotient - qCO_2, microbial quotient, and the activity of the enzymes urease, β-glucosidase, arylsulfatase, and acid phosphatase. The lowest values of most attributes were observed in the *Planosol* growing coffee. The activities of most of the enzymes were higher in the *Oxisol* under forest and lower in the *Planosol* under coffee. The microbiological attributes evaluated showed high correlation with soil organic matter. Different soil classes in more stable ecosystems, such as forests, may have comparable values of MBC, MBR, urease, and qCO_2. However, in coffee, there is a marked effect of the soil class on the values of these biological attributes. MBC, urease, β-glucosidase, and acid phosphatase are good soil quality indicators for coffee fields, which corroborates the results of another study in homogeneous environments in a *Oxisol*.

Keywords: Microbial biomass carbon, Microbial basal respiration, Soil enzymes, Atlantic Forest, *Coffea arabica* L.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was conducted in accordance with the ethical standards of the national research committee and/or comparable ethical standards.

Informed consent All the authors involved in this study consent to its publication.

Availability of data and material The dataset analyzed during the current study is available from the corresponding author upon reasonable request after the completion of the project.

Code availability Not applicable

Authors' contributions *Osnar Obede da Silva Aragão:* Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. *Ederson da Conceição Jesus:* Formal analysis, Investigation, Validation, Visualization, Writing - review & editing. *Silvia Maria de Oliveira-Longatti:* Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. *André Alves Souza:* Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. *Fatima Maria de Souza Moreira:* Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing, Funding acquisition, Project administration, Supervision.

Introduction

Brazil is the country of greatest production and export of coffee in the world. It leads this ranking with 1/3 of worldwide production, which is grown on 2.16 million hectares, with mean production of 3.57 million kg of coffee per year (CONAB 2020). Of the total planted area, 1.23 million hectares are in the state of Minas Gerais, with production of around 1.8 million kg of hulled coffee per year (CONAB 2020).

Conventional coffee growing in the state of Minas Gerais is based on intensive use of mechanization, fertilizers, and agricultural chemicals. This type of management of the production system can diminish the yield potential of growing areas over time due to loss of organic matter, soil compaction, and soil erosion. This process can lead to abandonment of these soils and migration of the coffee growing activity to new areas, including those of natural vegetation. Not coincidentally, the state of Minas Gerais has the highest rate of deforestation of the second largest Brazilian biome, the Atlantic Forest. In the last 10 years, 1.65 thousand hectares have been deforested (Fundação SOS Mata Atlântica and INPE 2019) and in 2018 and 2019, the rate of deforestation rose 27.2%, which makes this biome the most endangered in Brazil (Fundação SOS Mata Atlântica 2020). In spite of legal protection, the threats to this biome persist, especially through attempts at expansion of agricultural areas as a result of flexibilization of environmental laws.

Given the global importance of coffee growing and considering that the state of Minas Gerais is responsible for 52% of Brazilian coffee production (CONAB 2020), it is necessary to conciliate production of this crop with environmental protection. There is thus a pressing need to monitor, enhance, and/or maintain the quality of coffee field soils. Ensuring this quality will result in sustainable production from the soil and avoid expansion of agricultural lands to natural forest areas. This is indispensable, especially because the state has the largest area of natural forest (2.8 million hectares) of the Atlantic Forest biome (Fundação SOS Mata Atlântica and INPE 2019).

The need to preserve soils to ensure provision of their ecosystem services and sustained yield has awakened worldwide interest. Prominent among the objectives proposed by the United Nations for the 2030 agenda are practices that improve the quality

of the soil and the models of agricultural production (ONU 2015). Achieving these objectives requires an understanding of soil as a dynamic ecosystem, whose equilibrium is a result of harmonious operation of physical, chemical, and biological components. Microbiological components are vital for maintaining the soil because they act to generate energy through biochemical transformations and, since they are sensitive to changes that occur in the soil, they are considered early and effective indicators of its quality (Doran 1980; Doran and Parkin 1994). However, their value as indicators can vary according to the soil class and the type of plant cover, as shown in studies developed with other objectives (Acosta-Martínez et. 2008; Vinhal-Freitas et al. 2017)

Few studies have evaluated the biological quality of the soil in the Atlantic Forest domain in regard to coffee growing. Research found in the literature on this theme is mainly concerned with general microbiological attributes such as microbial carbon and macrofauna, with little exploration of soil enzymatic activities (Theodoro et al. 2003; Pimentel et al. 2006; Lammel et al. 2015). Recently, a more encompassing study (Aragão et al. 2020a) showed that microbiological attributes were more related to higher coffee yield than physical and chemical attributes. The authors showed that under homogeneous conditions (including edaphic conditions), microbial biomass carbon, qCO_2, and the enzymes urease and β-glucosidase had greatest importance in discrimination of higher yielding areas. In this respect, knowing the response of the microbiological attributes in coffee fields and adjacent forests formed over different soils is fundamental for monitoring the ecological quality of these environments. This knowledge is indispensable for finding quality indicators that can be applied under different edaphic conditions and for determining the need for interventions to maintain sustained production of the soils.

Our aim in this study was to determine the effect of different soil classes and plant covers (climax communities and agricultural systems) on microbiological attributes and the potential of these attributes as soil quality indicators for conventional coffee fields in the Atlantic Forest domain. The attributes were evaluated in *Oxisols* and *Planosols* under the hypothesis that the relevance of the biological attributes as quality indicators can vary among soil classes and plant covers.

Methodology

Characterization of the region and of the experimental areas

The study was conducted in the state of Minas Gerais in February 2019 in experimental areas and farms in the municipalities of Lavras and Itumirim in the

Southeast region of Brazil (Fig. 1a). The geographic coordinates of the area in Lavras are 21°14’06”S, 45°00’00’’W, and altitude of 918 masl; and in Itumirim are 21°15’57’S, 44°50’49’’W, and altitude of 870 masl. The climate of the region is classified as rainy temperate, with dry winter and wet summer. The predominant climate conditions in the period of soil sample collections are described below (Fig. 1b).

Two soil classes with contrasting physical characteristics (*Oxisol* and *Planosol*) were used in this study. These classes were chosen because they represent approximately 41% of Brazilian territory (Santos et al. 2011) and are highly used for coffee production. The first area of study was an experimental coffee (*Coffea arabica* L.) field, with soil composed of 50% clay, 23% silt, 27% sand, and 4.93 dag kg^{-1} organic matter, classified as a *Oxisol (Latossolo Vermelho Distrófico Típico* - Curi et al. 2017) on flat terrain. The second area was an irrigated field with production of specialty coffee, produced for export. This crop field was formed on a very sandy *Planosol* (*Planossolo Háplico* - Santos et al. 2018) composed of 15% clay, 6.4% silt, and 78% sand, with 2.0 dag kg^{-1} of organic matter. The third area was a forest formed on a soil composed of 36% clay, 20% silt, 44% sand, and high input of organic matter (7.3 dag kg^{-1}). The soil of this area is classified as a *Oxisol (Latossolo Vermelho-Amarelo Distrófico Típico* - Curi et al. 2017), also on flat terrain. This forest is a fragment that remains of the Atlantic Forest with open canopy of approximately 15 m height. It is classified as a seasonal semideciduous montane forest, and the main species are *Miconia cinnamomifolia, Piptadenia gonoacantha, Maprounea guianensis*, and *Cabralea canjerana* (D’angelo Neto et al. 1998). The fourth area was a forest fragment classified as a gallery forest (Loschi et al. 2013), with a steep slope in the direction of a stream. This forest was formed on a *Planosol (Planossolo Háplico* - Santos et al. 2018) with 18% clay, 17% silt, and 65% sand, and high organic matter content (6.4 dag kg^{-1}) in the first 10 cm of depth. The trees of this forest have an open canopy and are of approximately 10 m height, and the most representative species are *Protium spruceanum, Copaifera langsdorffii, Tapirira obtusa, Protium widgrenii, Machaerium villosum, Luehea candicans*, and *Myrcia tomentosa* (Loschi et al. 2013).

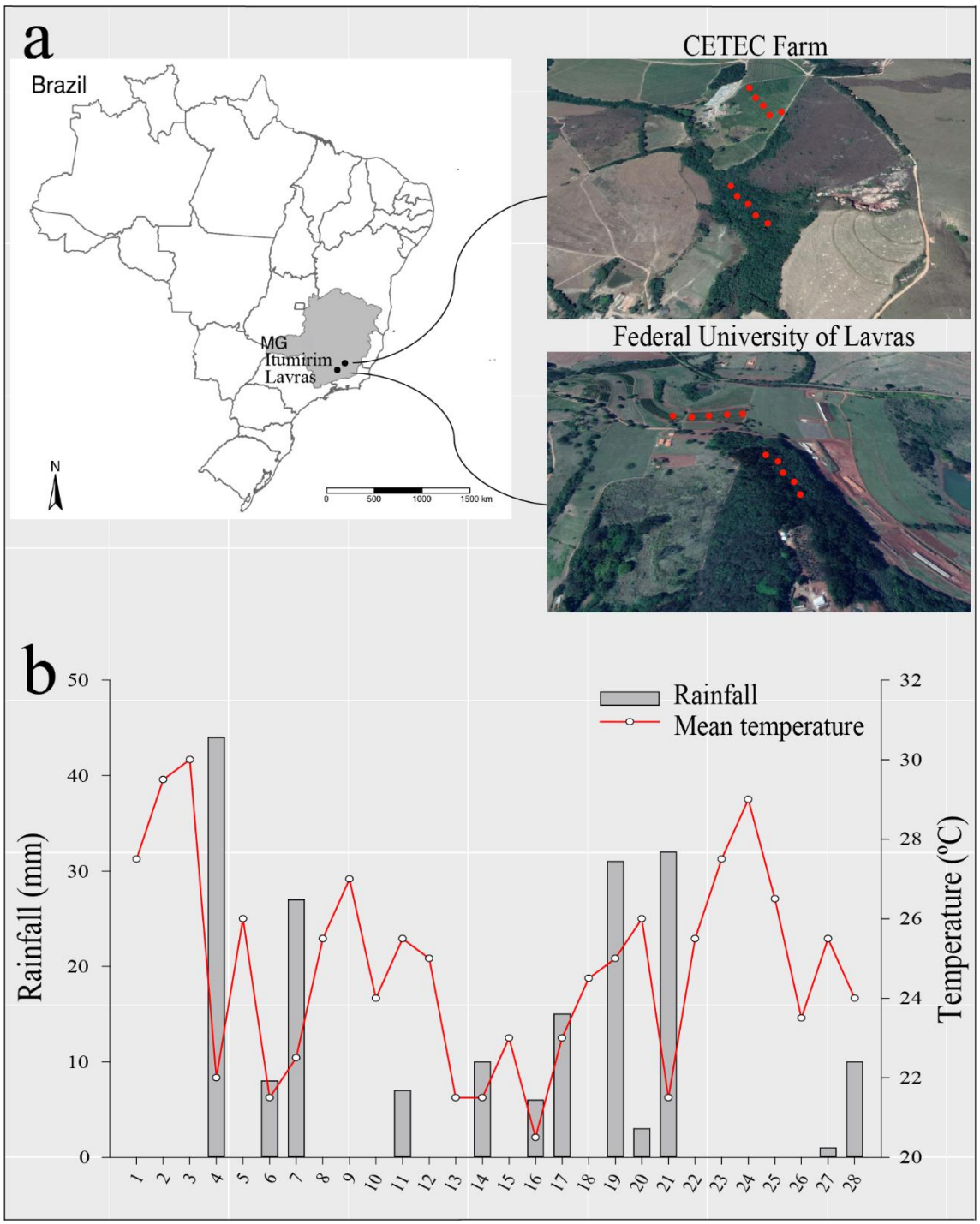

Fig. 1 (a) Satellite image showing the locations of soil sample collection in the areas studied in the municipalities of Lavras and Itumirim in Minas Gerais, Brazil. (b) Rainfall and mean temperature in February 2019 in the region under study.

Collection of soil samples

Field samplings for microbial biomass carbon, urease, β-glucosidase, arylsulfatase, fluorescein diacetate hydrolysis, microbial basal respiration, and acid phosphatase were carried out on February 4, 7, 10, 13, 16, 19, and 22, 2019. A three-day

interval between soil collections was used to ensure that each attribute was quantified 15 hours after field collection. Thus, an effect of storage time on the responses of the microbiological attributes would be avoided (Aragão et al. 2020b). Five compound samples (n = 5) spaced at 50 m from one another were obtained from each area. This distance was used to capture a significant amount of variability of the areas, compensate the effects of spatial autocorrelation, and minimize the sources of pseudoreplication (Hargrove and Pickering 1992; Mariotte et al. 1997; Ritz et al. 2004). The compound samples were formed from the mixture of four single samples collected in the area of a circle with radius of 1 m around a georeferenced point. A total of 20 compound samples were obtained (two soil classes × two types of plant cover × five replications). The soil samples were removed with the aid of a straight shovel to a depth of 10 cm after careful removal of plant litter. These samples were taken in plastic bags to the laboratory for processing at the Universidade Federal de Lavras. The soil with the same moisture content as in the field was sieved in 2 mm screens for removal of fine roots and other plant litter, and a 400 g aliquot was removed for physical and chemical analyses. The rest of the sample was weighed and stored at 4ºC for microbiological analyses on the morning of the following day, for a total of 15 hours between collection and analyses.

Physical and chemical analyses

The physical and chemical attributes evaluated were pH, phosphorus (P), remaining phosphorus (PRem), potassium (K), zinc (Zn), manganese (Mn), copper (Cu), calcium (Ca), magnesium (Mg), exchangeable aluminum (Al), active acidity (pH), potential acidity (H + Al), aluminum saturation percentage (m), exchangeable sum of bases (SB), base saturation (V), effective cation exchange capacity (t), potential cation exchange capacity (T), sulfur (S), organic matter (OM), and texture. These attributes were quantified according to the proposed methodologies of Mehlich (1953), Shoemaker et al. (1961), McLean et al. (1958), Hoeft et al. (1973), Walkley and Black (1934), and Bouyoucos (1951), and the results are shown in Table 1.

Table 1 Chemical and physical attributes of soil environments studied. (Aragão et al., 2020b)

soil environment	Chemical attributes								
	pH	P	K	Ca^{2+}	Mg^{2+}	S	Mn	Cu	Zn^{+2}
		------mg dm^{-3}-------		-----$cmol_c$ dm^{-3}-------		----------mg dm^{-3}----------			
Clay-forest	4.5 † ± 0.3††	4.2 ± 0.7	75 ± 7	3.2 ± 0.8	0.8 ± 0.2	4.7 ± 1.7	70.6 ± 23	2.9 ± 0.3	2 ± 0.4
Sand-forest	4.6 ± 0.3	11 ± 1.4	133 ± 60	1.4 ± 0.9	0.6 ± 0.3	3.3 ± 0.3	14.3 ± 8.8	0.8 ± 0.4	3 ± 0.4
Clay-coffee	4.8 ± 0.3	134 ± 32	583 ± 42	3.5 ± 0.2	0.7 ± 0	13 ± 3.1	12.0 ± 1.2	5.6 ± 0.4	16 ± 2
Sand-coffee	5.5 ± 0.1	351 ± 89	147 ± 46	1.5 ± 0.1	1.2 ± 0.1	1.4 ± 0.3	15.0 ± 2.3	6.3 ± 1.2	41 ± 34

soil environment	Chemical attributes						Physical attributes		
	SB	H+Al	T	V	m	OM	Clay	Silt	Sand
	----------$cmol_c$ dm^{-3}----------			---------%--------		dag kg^{-1}	---------- dag kg^{-1}----------		
Clay-forest	4.3 ± 1.0	10 ± 2.3	14 ± 3.2	29 ± 2	10 ± 3.3	7.3 ± 1.3	35 ± 4	20 ± 2	44 ± 6
Sand-forest	2.4 ± 1.1	17 ± 3.2	19 ± 3.1	12 ± 5	41 ± 17	6.4 ± 1.0	18 ± 3	16 ± 6	64 ± 5
Clay-coffee	5.7 ± 0.5	8.9 ± 2.1	14 ± 2.0	39 ± 7	2.7 ± 0.8	4.5 ± 0.5	49 ± 1	23 ± 1	27 ± 2
Sand-coffee	3.1 ± 0.3	3.3 ± 0.3	6 ± 0.4	48 ± 3	3.1 ± 0.3	2.0 ± 0.1	15 ± 1	6.4 ± 1	78 ± 1

†Means obtained from a value n = 5, ††Standard deviation. SB: sum of exchangeable bases, H + Al: potential acidity, T: cation exchange capacity at pH 7.0; V: base saturation, m: aluminum saturation, OM: organic matter.

Microbiological analyses

The following microbiological attributes were evaluated: microbial biomass carbon by the fumigation method with ethanol-free chloroform (Jenkinson and Powlson 1976) and carbon extraction with K_2SO_4, assuming a fumigation-extraction efficiency of 0.45 (Vance et al. 1987); microbial basal respiration by the estimate of CO_2 evolved during the incubation process (Jenkinson and Powlson 1976); fluorescein diacetate hydrolysis, determined by release of fluorescein (Dick, 1996); urease activity (EC 3.5.1.5) based on determination of ammonia released after incubation of the soil with urea solution (Tabatabai and Bremmer 1972); and β-glucosidase (EC 3.2.1.21), arylsulfatase (EC 3.1.6.1), and acid phosphatase (EC 3.1.3.2) activities, determined by colorimetry (Dick et al. 1996). A detailed methodological description of these analyses was presented in Aragão et al. (2020a).

Data analyses

The normality test (Shapiro-Wilk) was used on the data by the Past Statistics 1.0.0.0 program, and when they were not in conformity with the suppositions of the analysis, they were transformed in log at base 10. After meeting the statistical presuppositions, we used the permutational multivariate analysis of variance

(PERMANOVA) as hypothesis test. This analysis was used because it is the most robust and appropriate analysis for multivariate ecological data coming from non-classical experimental designs, whose responses can be simultaneous and of potentially non-independent variables (Anderson 2001). After that, principal component analyses (PCA) were performed from the covariance matrix of the original variables. The first two components (PC1 and PC2) were used to represent the total variability of the data, and the attributes with the highest eigenvectors were considered most important in discrimination of the areas. The PCA was used to cluster the variables in factors based on their correlation structure, and the groups identified in it were significant by PERMANOVA. The PCA with all variables was calculated following the nonlinear iterative partial least-squares algorithm (NIPALS, Algorithm 2.1) (Wold, 1966; Varmuza and Filzmoser, 2009). The PERMANOVA was calculated using the vegan package (Oksanen et al. 2018) in the R 3.3.1 programming language (R Core Team 2018). When significance was found by PERMANOVA, analysis of variance was carried out and the mean values of the attributes in the areas were separated by multiple comparison based on cluster analysis of mean values by the Scott-Knott algorithm at the $p \leq 0.05$ level of significance. The data were expressed in mean values, and the standard deviation was used as a measure of variability. The results of this analysis for the microbiological attributes are shown in bar graphs produced in the Sigma Plot 12.5 and Illustrator CC 2017 21.0.0 programs and in tables for the physical and chemical attributes. Finally, a correlation matrix was made to explore the relations between the microbiological attributes and soil organic matter.

Results

Permutational multivariate analysis of variance (PERMANOVA) and principal components (PCA) of the physical, chemical, and microbiological attributes

PERMANOVA identified that both the soil classes and the types of plant covers and their interactions were highly significant (Table 2). The PCA calculated based on microbiological attributes explained 81.43% of the variation of the data in the first two components (Fig. 2a). PC1, in particular, explained 65.63% of this variation and had high power of discrimination among the soil classes and types of plant covers studied. When only the microbiological attributes were analyzed, the FDA, microbial biomass carbon, and the enzymes urease, β-glucosidase, and acid phosphatase had greater importance in this discrimination (Table S1).

When calculated based on the physical and chemical attributes, PCA explained 74.77% of the total variation of the data and identified a gradient represented by pH, P, remaining P, Zn, Mg, Cu, V, potential CEC (T), H+Al, and organic matter in the first component (42.08%). This analysis shows a contrast of the forests and the coffee field on a *Oxisol* compared to the coffee field on a *Planosol* (Fig. 2b and Table S1). PC2 (32.69%) represents a gradient of attributes linked to fertility (Ca, K, S, B, SB, Al) and texture (clay and sand). Among these attributes, organic matter, T, H+Al, pH, P, and Zn exhibited the greatest weight in discrimination of the areas (Table S1).

The PCA that included all the attributes explained 68.76% of the total variation of the data in the first two components (Fig. 2c). The separation of the study areas was quite similar to that observed in the second PCA (Fig. 2b). Except for qCO_2 and *q*MIC, all the microbiological attributes were positively and significantly correlated with soil organic matter (Fig. 3).

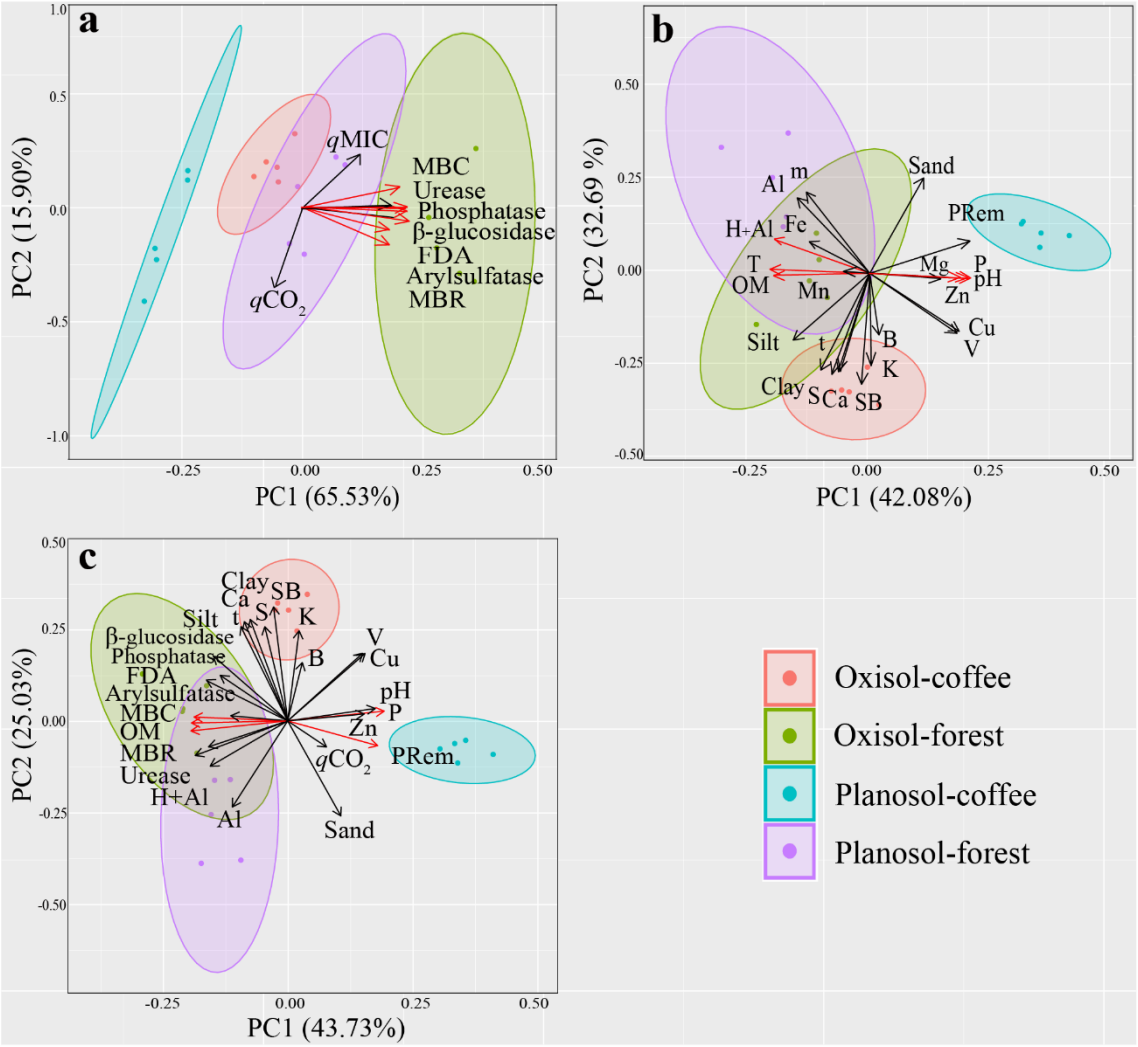

Fig. 2 Principal Component Analysis for (a) microbiological attributes, (b) physical and chemical attributes, and (c) physical, chemical, and microbiological attributes in the four areas studied. The red vectors represent the attributes of greatest importance in discrimination of the areas. OM: soil organic matter, MBC: microbial biomass carbon, MBR: microbial basal respiration, qCO_2: metabolic quotient, *q*MIC: microbial quotient, FDA: fluorescein diacetate hydrolysis.

Table 2 Permutational multivariate analysis (PERMANOVA) of variance of physical, chemical and microbiological atributes in soil clasess, plant covers and contrast Soil Class:Plant cover.

	Microbiological atributes					
Source	Df	SS	MS	model *F*	R^2	*p*-value
Plant cover	1	0.62	0.62	241	0.51	0,001***
Soi Class	1	0.39	0.39	152	0.32	0,001***
Soil Class : Plant cover	1	0.15	0.15	57	0.12	0,001***
Resídue	16	0.04	0.00		0.03	
Total	19	0.22			1.00	
	Physical-chemical atributes					
Fonte	Df	SS	MS	model *F*	R^2	*p*-value
Plant Cover	1	0.98	0.98	122	0.55	0,001***
Soil Class	1	0.37	0.37	46	0.29	0,001***
Soil Class : Plant cover	1	0.28	0.28	35	0.16	0,001***
Resídue	16	0.12	0.01		0.07	
Total	19	1.76			1.00	

Df: degrees of freedom, SS: sum of squares, MS: mean square. ***significant *p*-value ≤ 0,001.

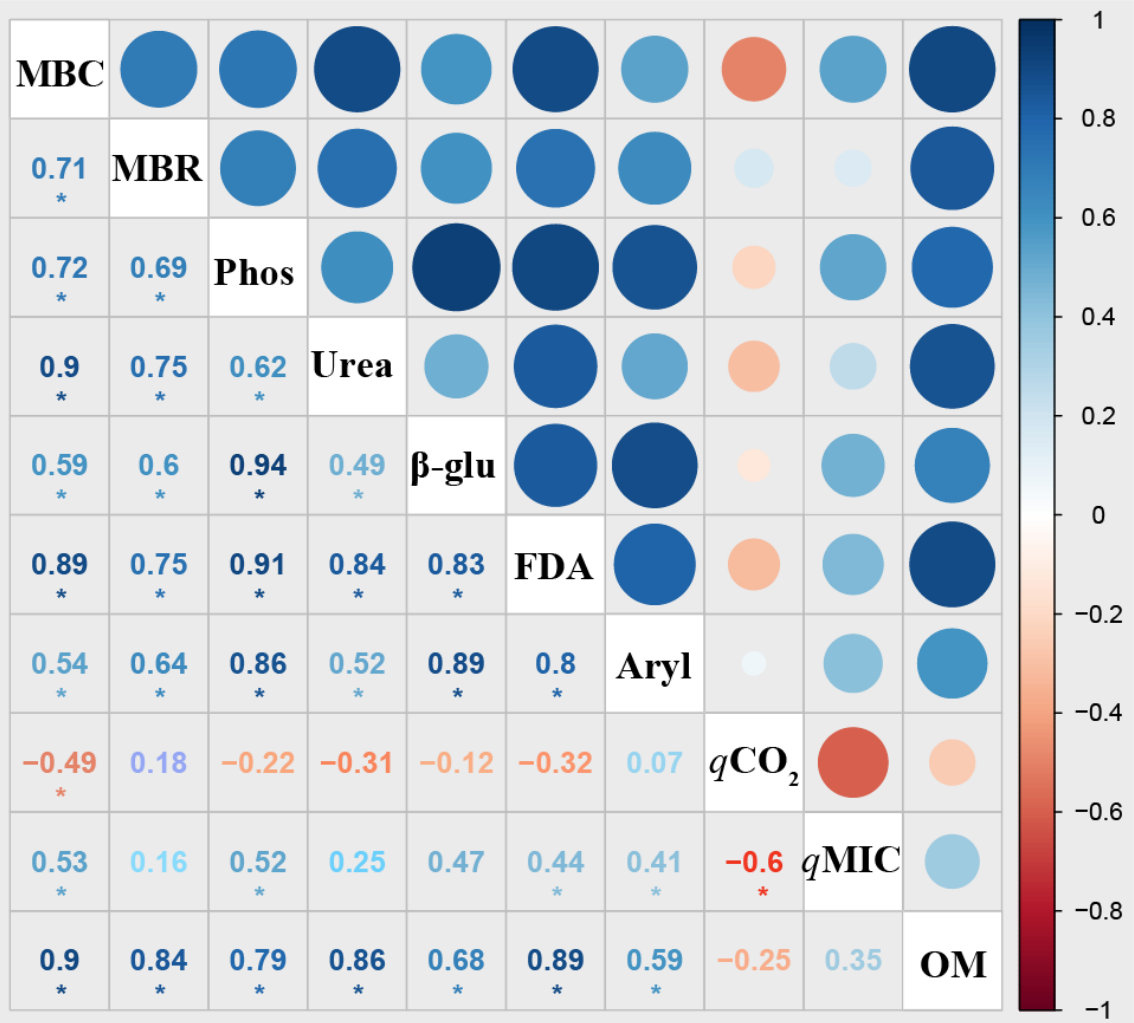

Fig. 3 Correlation matrix of the soil microbiological attributes and organic matter. MBC: microbial biomass carbon, MBR: microbial basal respiration, qCO_2: metabolic quotient, *q*MIC: microbial quotient, FDA: fluorescein diacetate hydrolysis, Phos: phosphatase, Ure: urease, β-glu: β-glucosidase, Aryl: arylsulfatase, OM: organic matter. * significant at $p \leq 0.05$.

Effect of the different soil classes and plant covers on microbiological attributes

In accordance with the significance observed in PERMANOVA, individual ANOVAs were carried out for each attribute. Comparison of the mean values showed that microbial biomass carbon, microbial basal respiration, and urease were similar in the soil classes from the forest areas, but higher than the values from the different soil classes in the coffee fields (Fig. 4a, 4b, and 4f). Lower values of microbial biomass carbon (374 mg C kg^{-1} dry soil) and microbial basal respiration (127 mg C-CO_2 kg^{-1} dry soil day^{-1}) were observed in the *Planosol* under coffee growing. This area had the highest metabolic

quotient (Fig. 4c), and the microbial quotient was similar among all the areas evaluated (Fig. 4d).

Enzymatic activity was greater in the *Oxisol* of the forest area, which had higher values of FDA and activities of the enzymes β-glucosidase, arylsulfatase, and acid phosphatase (Fig. 4e, 4g, 4h, and 4i). An exception was for urease activity (Fig. 4f), which did not differ between the forest areas. The values of FDA, urease, and acid phosphatase were lower in the *Planosol* under coffee growing, and the other areas had intermediate values of these enzymes. Arylsulfatase activity did not differ between the *Planosols* under forest and coffee growing.

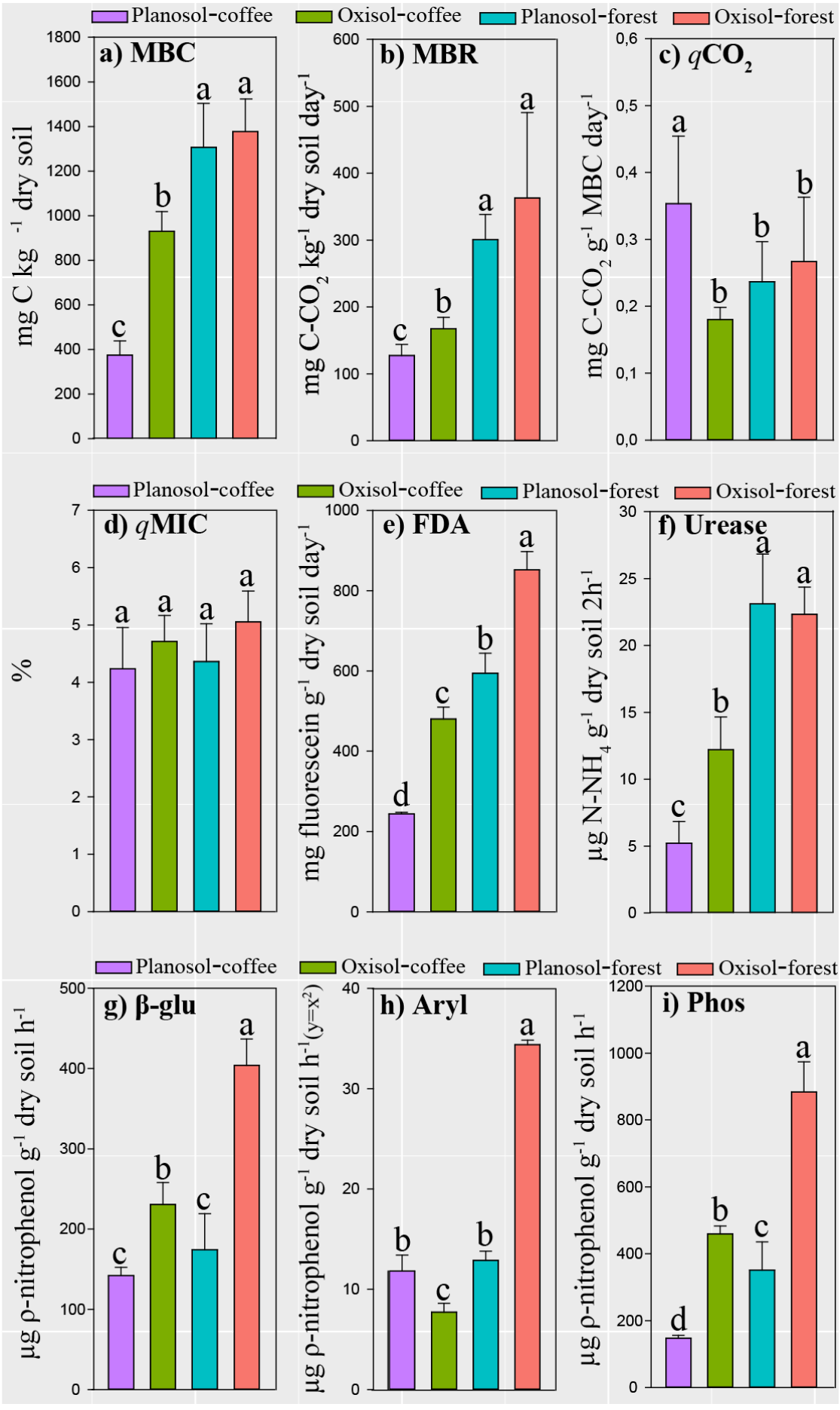
Planosol-coffee
Oxisol-coffee
Planosol-forest
Oxisol-forest
a) MBC
mg C kg -1 dry soil
b) MBR
mg C-CO2 kg-1 dry soil day-1
c) qCO2
mg C-CO2 g-1 MBC day-1
d) qMIC
%
e) FDA
mg fluorescein g-1 dry soil day-1
f) Urease
µg N-NH4 g-1 dry soil 2h-1
g) β-glu
µg ρ-nitrophenol g-1 dry soil h-1
h) Aryl
µg ρ-nitrophenol g-1 dry soil h-1(y=x2)
i) Phos
µg ρ-nitrophenol g-1 dry soil h-1

Fig. 4 Microbiological attributes of the four areas studied in the municipalities of Lavras and Itumirim, MG, Brazil. MBC: microbial biomass carbon; MBR: microbial basal respiration; *q*CO_2: metabolic quotient; *q*MIC: microbial quotient; FDA: fluorescein diacetate hydrolysis; β-glu: β-glucosidase; Aryl: arylsulfatase; Phos: phosphatase. The bars represent the mean values of five replications (n=5) and the letters compare the different areas by the Scott-Knott algorithm

Discussion

To interpret the values of the microbiological attributes as quality indicators of a determined soil, there must be reference values. These values can be obtained through evaluation of an adjacent natural environment or can come from an area with high yield (Doran and Parkin 1994; Bünemann et al. 2018). In the latter case, although the association between microbiological attributes and high coffee yield has been clearly shown, it was not possible to identify a cause/effect relationship between them (Aragão et al. 2020a). For that reason, we used the adjacent natural forests to observe the dynamic of the microbiological attributes in contrasting soil classes, but under more stable ecosystems. This would consequently allow an understanding of the effect of different soil classes on the relevance of these attributes as quality indicators in agricultural systems.

In this study, the superiority of the values for most of the microbiological attributes in the forests is probably due to the constant supply of carbon and to the quality of the organic matter added to the soil. In the literature, authors have reported higher values of microbial biomass carbon and microbial basal respiration in soils of the Atlantic Forest compared to conventional coffee fields (Partelli et al. 2012). These authors and other authors, however, did not observe differences between these attributes for forests and organic coffee growing (Theodoro et al. 2003). These results may be related to the addition of organic matter rich in nitrogen in these soils over long periods of time. In that regard, the values of microbial biomass carbon in the forests we studied were higher than in other studies in the Atlantic Forest (Pimentel et al. 2006, Partelli et al. 2012). This superiority may be related to the greater organic matter content in the soil. For example, the *Oxisol* and the *Planosol* of the forests we studied had 7.3 and 6.4 dag kg^{-1} organic matter, respectively, whereas the forests studied by the authors cited above had 2.7 and 2.9 dag kg^{-1}, respectively. These results show the importance of organic matter in maintaining the ecological balance of the soil. In a healthy forest, the abundance of plant

species promotes constant renewal of a diversity of organic compounds. The most labile compounds, with predominance of O-alkyl groups, serve as substrates for the fungal biomass that composes a large part of microbial biomass and grows rapidly under these conditions (Flanagan and Van Cleve 1983). Compounds of intermediate stability, such as lignin (Baldock et al. 1992), serve as substrates for r-strategist microorganisms, and those with predominance of aromatic groups, such as polymethylenes, serve as a source of energy for the K-strategists. Thus, the entire microbial community derives benefit from this environment and, therefore, it has high biomass and activity, regardless of the soil texture. Thus, the greater the availability of carbon in the soil, the greater the amount of energy for microorganisms and, therefore, the greater the number of microbial cells formed. The absence of significant differences in microbial biomass carbon, microbial basal respiration, urease, and qCO_2 between the forests (Fig. 4a, 4b, 4c, and 4f) indicates that under conditions that allow accumulation of organic matter, the microbial community can increase in a *Planosol*. This increase is sufficient to become comparable to a *Oxisol*, where there is greater protection and retention of nutrients because of the clay.

Between the coffee fields, the lower values for most of the microbiological attributes for the *Planosol* may be related to the lower capacity of this soil to protect organic matter. Lower physical protection and greater availability of oxygen can explain the greater microbial cycling in this soil, indicated by the qCO_2 (Van Veen et al. 1987). In the sandier texture, the number of microbial "microenvironments" is smaller, since they are associated with soil microporosity. In this respect, the greater qCO_2 in the *Planosol* soil under coffee growing may be associated with oscillation in the availability of water and nutrients, and rapid decomposition of organic matter by greater availability of oxygen and higher temperature (Anderson and Domsch 1990; Franzluebbers et al.1996; Muller and Hoper 2004). All these factors impose stress conditions on the community, reducing the number of microbial cells and increasing respiration per biomass unit, which results in higher qCO_2. Along with these factors is the disturbance caused by conventional soil management practices, which, associated with the lower supply of carbon, place stress on the microbial community. These results are consistent with those of other authors, which showed higher values of qCO_2 in traditional agricultural areas in contrast with natural, revegetated, or organic production areas in the Atlantic Forest domain (Partelli et al. 2012; Nogueira et al. 2016; Tavares et al. 2018). The results are also in agreement with those presented by Aragão et al. (2020a), who observed higher values of qCO_2 for lower yielding coffee fields in the Brazilian Cerrado

(tropical savanna). Therefore, the higher values of qCO_2 in the *Planosol* under coffee growing indicate that there is greater susceptibility to conventional management practices in that soil.

Higher values of FDA and activity of the enzymes β-glucosidase, arylsulfatase, and acid phosphatase in the clayey soil under forest may be due to the role of mineral fractions in protection of these enzymes. In that environment, the predominance of the mineral fractions in the silt and clay particle size (2-50 and < 2 μm, respectively) may have promoted greater protection against enzymatic biodegradation. These enzymes are mainly extracellular and, therefore, more subject to the pressures imposed by the environment (McGill Colle 1981; Bhatia et al. 2002). In this respect, the soils with a greater silt-clay proportion, because of their lower fluctuation in temperature, pH, and water availability, may have protected the enzymes from denaturation, increasing their stability and survival in the soil (Curtin and Rostad 1997; Bauhus and Khanna 1999). Our results indicate that the enzyme × texture interaction is more direct and prominent than the microbial biomass × texture interaction. That can likely result in an effect of texture on the dynamic of enzymes as indicators of soil quality. Nevertheless, definitive and unequivocal inferences in this respect require greater investigations. Even so, this is an aspect that cannot be ignored. However, the difficulty in applying a classical experimental design capable of isolating factors in field studies in perennial crops increases the need for studies on a broader geographical scale.

The superiority of the forest values for most of the microbiological attributes (Fig. 4) suggests an impact from conversion of these environments on the soil microbial community. Although these microorganisms constitute only a small portion of the soil organic matter (Smith and Paul 1990) as indicated by the *q*MIC (4-5%) found in this study (Fig. 4d), they are vital in maintaining soil health and in the organic matter decomposition process. This process is closely connected with the dynamics of carbon, energy production, and biogeochemical cycling of nitrogen, phosphorus, sulfur, and other elements (Hu et al. 1997). These aspects determine the good fertility, operation, and sustained production of the soil. In other words, the high values of the microbiological attributes are consistent with the state of greater ecological balance in the forest soils.

Except for acid phosphatase and FDA, microbial biomass carbon and the enzymes urease and β-glucosidase, were the ones that discriminated the areas evaluated in this study under heterogenous conditions. Aragão et al. (2020a) also found microbial biomass carbon and the enzymes urease and β-glucosidase discriminating the highest

yielding from the lowest yielding coffee areas, however, under homogeneous conditions. Results of Aragão et al. (2020a) do not agree with results of the present study regarding FDA. The importance of FDA as an indicator may be related to the diversity of organic substrates existing in systems with greater plant diversity, such as forests, compared to that of a monocrop such as coffee which could explain these different findings. FDA represents a pool of enzymes (proteases, lipases, and esterases) and, therefore, it shows the capacity to transform a greater diversity of substrates (Schnurer and Rosswall 1982). In contrast, qCO_2, relevant for discriminating high yielding areas under homogeneous conditions (Aragão et al. 2020a), had lower relevance in this study. Nevertheless, in choosing microbiological attributes as indicators of soil quality, storage time must be considered. It was recently shown that storing soil for long periods at 4ºC significantly reduces the values of the microbiological attributes (Aragão et al. 2020b). Considering that the PCA is obtained through the covariance matrix of the original variables (Wold et al. 1987), prolonged storage can reduce their eigenvectors and, consequently, the relevance of the attributes as soil quality indicators.

This study shows the need to use more sustainable management practices in soils growing coffee, especially sandier soils, and reinforces the role of the Atlantic Forest in protection of the soil microbial community. Management practices in coffee growing should be directed to enhancing the processes of gain in organic matter and/or reducing the processes of loss. The positive correlation of the microbiological attributes with organic matter indicates its importance in maintaining the biochemical processes of the soil that ensure soil quality. In this regard, agricultural practices capable of capturing and retaining carbon are fundamental for the quality of the soil of coffee fields in the Atlantic Forest domain. In addition, this study contributes to finding soil quality indicators that can be applied under heterogeneous edaphic conditions.

Conclusions

(a) In the coffee fields studied, the microbiological attributes had lower values in the *Planosol*, indicating that this soil may be under a state of lower ecological balance. (b) The high correlation of the microbiological attributes with organic matter indicates its importance in maintaining the biochemical processes of the soil that ensure soil quality. (c) Among the physical and chemical attributes, organic matter content, potential acidity, potential cation exchange capacity, pH, phosphorus, and zinc were most important in discrimination of the areas. (d) Different soil classes under adequate conditions, in the

case of more stable ecosystems such as forests, can have comparable values of microbial biomass carbon, microbial basal respiration, urease, and qCO_2. (e) In contrast, coffee fields had more discrepant values for all the attributes, indicating the more drastic effect of changes on the chemical and physical attributes of the soil. (f) FDA and the β-glucosidase and acid phosphatase enzymes were most affected by the different soil classes. (g) Microbial biomass carbon, FDA, urease, β-glucosidase, and acid phosphatase exhibited greater capacity in discrimination of the areas, showing that these attributes are good soil quality indicators for coffee growing areas.

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Supplementary material

Table S1 Eigenvectors of Principal component analysis – PCA-Fig. 2a - microbiological attributes, Fig. 2b - physical-chemical attributes, Fig. 2c - physical-chemical and microbiological attributes of the four studied areas.

Microbiological attributes					
	PC1	PC2		PC1	PC2
MBC[1]	0.37	0.18	FDA[3]	0.40	0.01
MBR[2]	0.32	-0.35	Arilsulfatase	0.34	-0.25
Phosphatase	0.39	-0.06	qCO_2[4]	-0.13	-0.74
Urease	0.35	0.01	*q*MIC[5]	0.23	0.49
β-glucosidase	0.36	-0.12			
Physical-chemical attributes					
	PC1	PC2		PC1	PC2
OM[6]	0.28	0.01	m[11]	0.18	-0.25
pH	-0.29	-0.01	P-Rem[12]	-0.29	-0.10
K	-0.00	0.29	Zn	-0.24	0.01
P	-0.29	0.01	Fe	0.18	-0.10
Ca	0.08	0.31	Mn	0.07	0.00
Mg	-0.21	0.01	Cu	-0.25	0.19
Al	0.21	-0.23	B	-0.02	0.19
H+Al	0.28	0.10	S	0.09	0.30
SB[7]	-0.02	0.35	Clay	0.11	0.32
t[8]	0.14	0.30	Silt	0.22	0.21
T[9]	0.29	0.01	Sand	-0.16	-0.30
V[10]	-0.28	0.18			
Physical-chemical and microbiological attributes					
	PC1	PC2		PC1	PC2
MBC	-0.25	-0.03	Al	-0.14	-0.25
MBR	-0.20	-0.08	H+Al	-0.20	-0.13
Phosphatase	-0.21	0.21	SB	-0.03	0.33
Urease	-0.24	-0.10	t	-0.12	0.28
β-glucosidase	-0.17	0.13	T	-0.22	-0.04
FDA	-0.24	0.01	V	0.20	0.20
Arylsulfatase	-0.15	0.01	P-Rem	0.23	-0.07
qCO_2	0.11	-0.11	Zn	0.20	0.02
*q*MIC	-0.10	0.11	Cu	0.20	0.20
OM	-0.25	-0.01	B	0.04	0.21
pH	0.23	0.03	S	-0.06	0.28

K	0.03	0.27	Argila	-0.10	0.30
P	0.25	0.03	Silte	-0.20	0.20
Ca	-0.10	0.30	Areia	0.14	-0.30

[1]MBC- Microbial biomass carbon, [2] MBR-Microbial basal respiration, [3]FDA-fluorescein diacetate hydrolysis, [4] *q*CO_2metabolic quotient, [5] *q*micmicrobial quotient. [6]OM-Organic matter, [7]SB: sum of bases, [8]t: cátion exchenage capacity, [9]T: potential cátion exchenage capacity,[10] V: Base saturation, [11]m: percentage of Al saturation, [12]P-Rem: Remaining.

Considerações finais

Todos os aspectos tratados neste estudo fazem parte de um conjunto de esforços que permitirão o monitoramento da qualidade dos solos. Neste sentido, nós buscamos elucidar a influência do tempo de armazenamento a 4 ºC na efetividade dos atributos microbiológicos utilizados como indicadores da qualidade de solos tropicais. Observamos que o armazenamento por longos períodos afeta significativamente os dados gerados e pode alterar a forma como são interpretados, levando a conclusões errôneas e comprometendo os avanços na utilização desses atributos. Por exemplo, em uma análise de componentes principais, o prolongamento do tempo de armazenamento pode reduzir os autovetores e, consequentemente, a relevância dos atributos como indicadores de qualidade (Figura 1 e Tabela 1). Portanto, nós chamamos a atenção para esse aspecto e propomos uma ordem de prioridade para a quantificação de cada atributo. Isso dará maior flexibilidade à realização de análises, especialmente nos casos em que for necessário processar muitas amostras.

Com relação ao efeito de diferentes classes de solo sobre os atributos microbiológicos e o potencial desses atributos como indicadores da qualidade de diferentes solos, foi observado: a) diferentes classes de solo sob condições que permitem acumular matéria orgânica, como em florestas, podem ter valores equiparáveis de carbono da biomassa microbiana, respiração basal microbiana, urease e *q*CO_2; b) O carbono da biomassa microbiana, FDA, urease, β-glucosidase e fosfatase ácida exibiram maior capacidade de discriminação das áreas mostrando que esses atributos são bons indicadores da qualidade dos solos cultivados com café.

Finalmente, sugerimos que mais trabalhos críticos de aspectos práticos sobre procedimentos metodológicos sejam realizados. Isso elucidará muitas questões indefinidas e poderá maximizar a utilização desses atributos como ferramentas de monitoramento da qualidade dos solos.

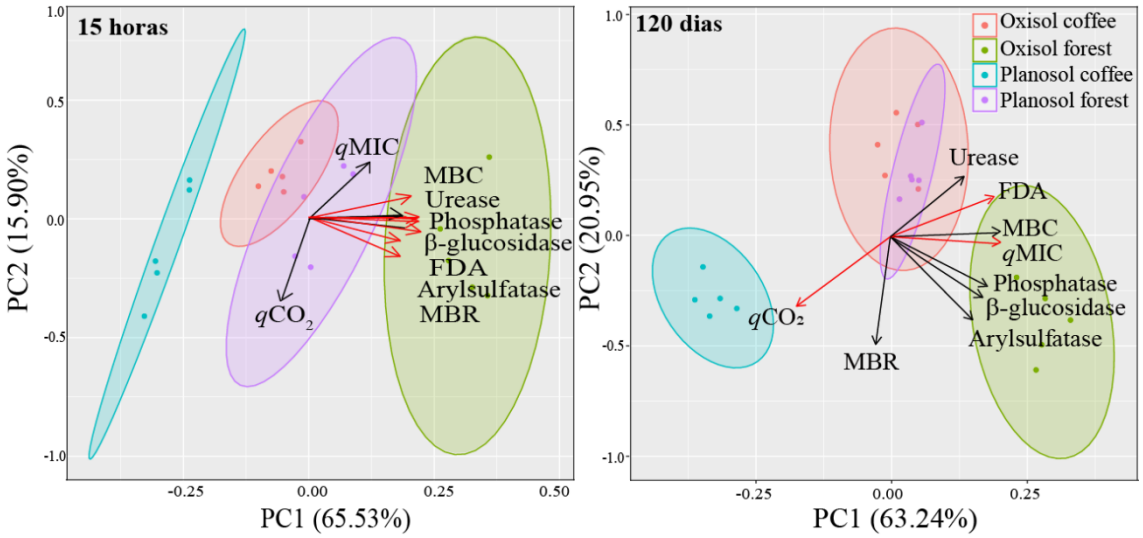

Fig. 3. Análises de componentes principais dos atributos microbiológicos em 15 horas e 120 dias de armazenamento do solo. MBC: carbono da biomassa microbiana, MBR: respiração basal microbiana, *q*CO_2: quociente metabólico, *q*MIC: quociente microbiano, FDA: hidrólise do diacetato de fluoresceína.

Tabela 1: Autos vetores das PCA's de 15 horas e 120 dias dos atributos microbiológicos

Atributos microbiológicos 15h					
Variáveis	PC1	PC2	Variáveis	PC1	PC2
MBC	0.37	0.18	FDA	0.40	0.01
MBR	0.32	-0.35	Arilsulfatase	0.34	-0.25
Fosfatase	0.39	-0.06	*q*CO_2	-0.13	-0.74
Urease	0.35	0.01	*q*MIC	0.23	0.49
β-glucosidase	0.36	-0.12			
Atributos microbiológicos 120d					
Variáveis	PC1	PC2	Variáveis	PC1	PC2
MBC	0.39	0.02	FDA	0.37	0.21
MBR	-0.05	-0.59	Arilsulfatase	0.30	-0.45
Fosfatase	0.35	-0.26	*q*CO_2	-0.34	-0.38
Urease	0.27	0.33	*q*MIC	0.40	-0.03
β-glucosidase	0.35	-0.27			

MBC: carbono da biomassa microbiana, MBR: respiração basal microbiana, *q*CO_2: quociente metabólico, *q*MIC: quociente microbiano, FDA: hidrólise do diacetato de fluoresceína.