MARIANE PERTILE

RESPOSTAS DA COMUNIDADE MICROBIANA A APLICAÇÃO DOS HERBICIDAS IMAZETHAPYR E FLUMIOXAZIN.

TERESINA – PI

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Orientador
Dr. Ademir Sérgio Ferreira de Araújo

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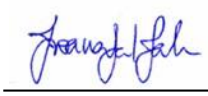

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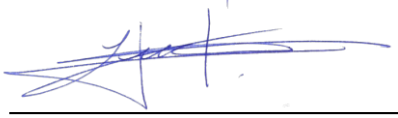

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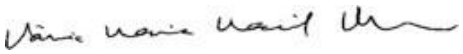

Profa. Dra. Vânia Maria Maciel Melo – UFC

Prof. Dr. Ademir Sérgio Ferreira de Araújo – CCA/UFPI
(Orientador)

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RESUMO GERAL

O manejo químico com herbicidas é o método mais utilizado no controle de plantas daninhas em áreas agrícolas comerciais. No entanto, seu uso pode gerar impactos sobre os microrganismos do solo. Desta forma, os objetivos da pesquisa foram avaliar a resposta da biomassa microbiana, atividade enzimática e comunidade bacteriana do solo à aplicação dos herbicidas imazethapyr e flumioxazin. Em laboratório, amostras de solo, oriundas de áreas agrícolas, foram incubadas e submetidas a aplicação isolada ou associada de imazethapyr e flumioxazin. Os efeitos dos herbicidas foram avaliados aos 0, 15, 30 e 60 dias após aplicação (DAA), onde verificou-se o carbono da biomassa microbiana (CBM), nitrogênio da biomassa microbiana (NBM), respiração basal (RB), quociente metabólico (qCo_2), carbono orgânico total (COT), quociente microbiano (qMic), relação CBM:NBM, atividade da desidrogenase (DHA), hidrólise do diacetato de fluoresceína (FDA). Realizou-se ainda a análise da comunidade bacteriana presente no solo utilizando-se a técnica de sequenciamento de nova geração. Os resultados demonstraram que em relação ao controle houve uma redução na quantidade do CBM, e um aumento significativo na RB, no qCo_2 e na atividade da DHA. No entanto, o NBM não foi afetado pela aplicação dos herbicidas. A FDA não diferiu entre os controles e os tratamentos com os herbicidas. A curva principal de resposta demonstrou que o flumioxazin possui um efeito inicial maior que o imazethapyr e sua mistura. O flumioxazin exerceu uma influência mais significa na RB e qCo_2 em relação aos demais herbicidas avaliados. A aplicação dos herbicidas alterou a abundância de alguns filos microbianos em específicos, onde verificou-se uma redução significativa de Acidobacteria, Verrucomicrobia, Elusimicrobia e Planctomycetes, e o aumento de Gemmatimonadetes, Bacteroidetes, Firmicutes e Proteobacteria. A Análise de componentes principais indicou que o tempo de exposição aos herbicidas influenciou as respostas das comunidades e na diversidade microbiana verificando-se um crescimento aos 15 dias, e uma tendência de redução aos 30 e 60 dias. Na avaliação das funções potenciais evidenciou-se uma maior representatividade quimioheterotrófica (35,1%), seguida pela quimioheterotrófica aeróbia (32,8%), nitrificação (6,2%), oxidação da amônia (6,2%) e degradação de compostos aromáticos (4,4%). Os dados ainda revelaram um acréscimo de grupos microbianos com potencial de metabolizar compostos químicos, como os gêneros *Bryobacter, Gaiella* e *Flavobacterium*, que aumentaram significativamente em abundância e podem ser explorados para uso biotecnológico no futuro. Por fim, o efeito dos herbicidas na biomassa microbiana e na atividade enzimática do solo foram de curto prazo, onde os parâmetros biológicos possuem a capacidade de recuperação ao longo do tempo. A aplicação herbicida afeta o perfil e as funções microbianas do solo, e esse efeito está relacionado a grupos com potencial de degradar compostos químicos.

Palavras-chave: Agrotóxicos, Microbiota, Solo, Sequenciamento de nova geração

ABSTRACT

Chemical herbicide management is the most widely used method for weed control in commercial agricultural. However, they can impact the soil microbiome. Thus the reseach objectives were evaluated the response of microbial biomass, enzyme activity and soil bacterial community to the application of the herbicides imazethapyr and flumioxazin. In the laboratory, soil samples were incubated with the application of imazethapyr and flumioxazin alone or in combination and analyzed at 0, 15, 30 and 60 days after application (DAA). Herbicide effects were evaluated for the Soil microbial biomass C (MBC), Microbial biomass N (MBN), basal respiration (RB), respiratory quotient (qCo_2), total organic carbon (COT), microbial quotient (qMic), relation CBM:NBM, dehydrogenase activity (DHA); hydrolysis of fluorescein diacetate (FDA). As well as the bacterial community present in the soil, using next-generation sequencing. The results demonstrated that the MBC decreased, while MBN was not affected after the application of the herbicides as compared to the control. Soil respiration, qCo_2, and DHA activity increased significantly after the application of the herbicides compared to the control. The FDA was not significantly different between the control and the herbicide treatments. The main response curve showed the largest initial effects for the flumioxazin, followed by imazethapyr and their mixture. Flumioxazin had a different influence on soil respiration and respiratory quotient than imazethapyr and their mixture. The abundance of specific microbial phyla changed as a response to the herbicide application with a significant decrease of Acidobacteria, Verrucomicrobia, Elusimicrobia, and Planctomycetes, and an increase of Gemmatimonadetes, Bacteroidetes, Firmicutes, and Proteobacteria. The principal component analysis clustered the samples according to the time of incubation and the microbial diversity increased on the 15th day with a trend to decrease at 30 and 60 days. The evaluation of potential functions showed core functions represented by chemoheterotrophy (35.1%), followed by aerobic chemoheterotrophy (32.8%), nitrification (6.2%), ammonia oxidation (6.2%), and aromatic compound degradation (4.4%). Our data showed an increase of microbial groups that have the potential to metabolize chemical compounds, such as the genera *Bryobacter, Gaiella*, and *Flavobacterium* which increased significantly in abundance and can be explored for future biotechnological use. The The results demonstrate that the effects of herbicides on soil microbial biomass and enzymes are short-term as we observed recovery in the biological parameters over time. The application of the herbicides in soil affects the microbial profile with the potential to affect functions mediated by microbial communities, and this effect is related to groups with the potential to degrade the compound.

Key words: Pesticidas, Microbiota, Soil, Next-Generation Sequencing.

SUMÁRIO

1. INTRODUÇÃO GERAL

O manejo químico com herbicidas é o método mais utilizado a nível mundial no controle de plantas daninhas em sistemas de produção agrícola comercial, em razão da sua rápida ação e eficiência de controle de infestantes (Fernando et al., 2016). No Brasil, eles respondem por 60% dos agrotóxicos comercializados (Pignati et al., 2017), sendo aproximadamente 22% destinado ao manejo da soja (*Glycine max* (L.) Merrill) (Schneider et al., 2020).

Dentre os herbicidas registrados para cultura da soja merecem destaque o imazethapyr e flumioxazin, seja em associação ou isolados, devido a eficiência desses produtos no manejo de plantas daninhas resistentes ou de difícil controle (Norsworthy, 2012; Melo, 2017; Coradin, 2019). O Imazethapyr, pertencente ao grupo das imidazolinonas, atua sobre plantas daninhas de folha larga e estreita inibindo a enzima acetolactato sintase (ALS) (Cantwell et al., 1989; Rodrigues; Almeida, 2011). Já, o flumioxazin, pertence ao grupo das ciclohexenodicarboximida, atua no controle de folha larga inibindo a enzima protoporfirinogênio oxidase (PROTOX) (Rodrigues; Almeida, 2005).

Embora o uso de herbicidas seja importante para o sistema agrícola, há uma preocupação com o seu destino no meio ambiente e, o seu impacto sobre os microrganismos do solo, especificamente nas propriedades biológicas (Zabaloy et al., 2011). Essas propriedades são extremamente importantes uma vez, que atuam em diversos processos chaves do solo, como a degradação de compostos químicos, tais como os herbicidas (Araujo; Monteiro; Abarkeli, 2003).

A biomassa microbiana e as atividades enzimáticas do solo são propriedades biológicas importantes e responsivas que são frequentemente indicadas para avaliar o efeito da aplicação de herbicidas nos microrganismos do solo (Pose-Juan et al., 2017). Por exemplo, Perucci e Scarponi (1994), em estudos realizados com o herbicida imazethapyr, não observaram seu efeito sobre a biomassa microbiana do solo utilizando a dose recomendada para a cultura da soja (1,6 mg kg^{-1}). Por outro lado, Zhang et al., (2010), constataram efeitos adversos sobre a biomassa microbiana do solo, ao utilizar o imazethapyr (0,1, 1 ou 10 $mgkg^{-1}$ solo) em experimentos à campo conduzido por dois anos. Esses resultados sugerem que o histórico de aplicação pode influenciar a biomassa microbiana, bem como a atividade enzimática do solo.

Em contrapartida ao imazethapyr, não há relatos na literatura sobre o efeito do flumioxazin isolado ou associado na biomassa microbiana e atividade enzimática do solo, sendo seus estudos voltados à sua dissipação, persistência e movimento no solo (Alister, 2008; Ferrell; Vencill, 2003). No entanto, estudar somente as propriedades biológicas pode oculta o real feito do uso de herbicidas sobre as comunidades microbianas do solo, tais como a bacteriana.

As bactérias desempenham papéis importantes no ecossistema do solo e são responsáveis por processos como a decomposição da matéria orgânica e ciclagem de nutrientes (Miranda, 2018), sendo fundamentais na renovação do carbono (C) e do nitrogênio (N) no ambiente terrestre (Bengtson; Sterngren; Rousk, 2012). Essas funções evidenciam a importância da diversidade e estrutura da comunidade bacteriana na atividade do solo (Hansel et al., 2008) que pode sofrer influência da aplicação de herbicidas.

O sequenciamento de última geração, baseado no sequenciamento do gene 16S rRNA tem sido o principal método de avaliação de comunidades bacterianas (Miranda et al., 2018). Essa técnica tem sido empregada com o objetivo de estudar o efeito de herbicidas na comunidade bacteriana que tem demonstrado efeitos negativos (Moretto et al., 2017), positivos (Crouzet et al., 2010) ou neutros (Dennis et al., 2018) dependendo do herbicida aplicado. Dennis et al., (2018) avaliaram o efeito do glifosato, glufosinato, paraquat e paraquat-diquat no solo e constataram que esses herbicidas não afetaram a diversidade e estrutura microbiana do solo. Por outro lado, Moretto et al., (2017) relataram que atrazina, diuron e 2,4-D alteraram a composição da comunidade microbiana.

Embora o emprego de imazethapyr e flumiozaxin seja uma importante opção no manejo de daninhas, ainda não há comprovação cientifica sobre a consequência da aplicação associada desses herbicidas e de forma isolada do flumioxazin, bem como seus efeitos na microbiota do solo ao longo do tempo.

Neste sentido, as questões cientificas podem ser construídas:

1) O histórico da aplicação de herbicida e seus diferentes modos de ação podem influenciar a biomassa microbiana e a atividade enzimática do solo?
2) A associação de herbicidas pode apresentar comportamento distinto em comparação com o composto isolado?

3) Distintos herbicidas podem apresentar diferentes efeitos na diversidade, estrutura e composição bacteriana do solo quando aplicados de forma isolada ou associados?

Com base no exposto, este estudo foi dividido em dois capítulos com o objetivo de:

Capítulo I **–** Avaliar as respostas da biomassa microbiana e atividade enzimatica do solo à aplicação de Imazethapyr e flumiozaxin de forma isolada ou associada em solo tropical**;**

Capítulo II – Verificar o comportamento da comunidade bácteriana à aplicação isolada ou associada dos herbicidas Imazethapyr e flumiozaxin no solo.

2. REFERENCIAL TEÓRICO

2.1 Os herbicidas

Os herbicidas são substâncias químicas biologicamente ativas (Baboo et al., 2013) utilizadas no controle de plantas daninhas. O seu uso configura-se como importante fator de cultivo em lavouras comerciais onde são amplamente utilizados e do qual depende o sucesso da produção (Childs et al., 2007; Zaboloy et al., 2011).

O manejo químico com herbicida no controle de plantas daninhas possui algumas vantagens, como a sua rápida ação, baixo custo e eficiência de controle em diferentes épocas de aplicação (Perroti et al., 2019; Fernando et al., 2016; Chauhan et al., 2012). Entretanto, algumas desvantagens também são observadas, como a necessidade de conhecimento em tecnologia de aplicação, seleção de biotipos daninhos resistentes e intoxicação de organismos não-alvo (Maciel, 2010).

Os herbicidas podem ser classificados de várias formas pela seletividade, translocação, mecanismo de ação e época de aplicação (Silva; Ferreira; Ferreira 2009b). A seletividade é a capacidade de eliminar plantas infestantes sem prejudicar a cultura, já os não seletivos atuam de forma indiscriminada sobre todas as espécies. Em ambas as classificações, os herbicidas matam por contato ou por meios mais complexos, após a absorção do produto pela planta (Lorenzi, 2014). A translocação leva em consideração duas classificações: sistêmicos e não sistêmicos, onde o primeiro possui a capacidade de se distribuir dentro das plantas e o segundo não.

O mecanismo de ação é a maneira mais utilizada para se classificar os herbicidas, visto que evidencia a forma de atuação dos produtos ativos. Corresponde ao ponto exato do metabolismo da planta em que o herbicida atua, o qual desencadeia uma série de eventos metabólicos que resultam na sua expressão final sobre a planta. Já o conjunto de eventos metabólicos e os sintomas causados recebem a denominação de modo de ação (Oliveira Júnior, 2001).

Em relação a época de aplicação, os herbicidas podem ser separados de acordo com o seu uso em três categorias como: pré-plantio incorporado (PPI), pré-emergência e pós-emergência. O PPI engloba os herbicidas aplicados diretamente no solo, os quais exigem uma posterior incorporação, que pode ocorrer de forma mecânica ou por irrigação. Os Pré-emergência tem como alvo o solo e, portanto, são indicados para a aplicação logo após a semeadura da cultura principal, mas antes da

sua emergência. Já, os Pós-emergência são indicados para aplicação depois da emergência da planta cultivada (Oliveira Jr, 2001).

Ao selecionar o tipo de herbicida, bem como a dose a ser empregada, o produtor deve estar atento a fatores como, tipo de espécies presentes na área, o estádio de desenvolvimento e a cultura subsequente a ser implantada (Buzatti, 1999). É fundamental que o seu uso aconteça em locais onde ocorre alta infestação de plantas daninhas, como se observa em culturas de grande importância agrícola como a soja. Os herbicidas tem se tornado o principal método empregado nas plantações de soja devido a sua agilidade e a eficiência no controle químico, gerando uma menor perda da produtividade (Gazziero; Vargas; Roman, 2004).

2.2 O emprego de herbicidas no manejo de plantas daninhas na cultura da soja e seu destino no ambiente.

As plantas daninhas constituem um dos principais componentes bióticos do agroecossistema da soja e, quando não manejadas, afetam negativamente o desenvolvimento da cultura, geralmente causando a redução na produtividade de grãos (Lamego et al., 2004). Além disso, competem com a cultura pelos recursos do meio, principalmente água, luz e nutrientes, liberando substâncias alelopáticas prejudiciais, atuando como hospedeiras de pragas e doenças comuns à cultura e interferindo nas práticas de colheita (Pitelli, 1985).

De acordo com Datta et al., (2017), a presença de daninhas durante a fase crítica de desenvolvimento da soja pode causar a redução de 8 a 55% na produtividade. Já Zandoná et al., (2018), afirma que esses valores podem ser ainda maiores (80%), caso medidas de controle não sejam empregados.

Vários métodos de controle estão disponíveis para o manejo de infestantes, sendo o controle químico com herbicida o mais adotado, respondendo por aproximadamente a 10% dos custos (IMEA, 2020). Só para soja existem 45 moléculas herbicidas registradas para uso (MAPA, 2021), onde sem dúvida o glyphosate é o mais adotado pelos agricultores.

Entretanto, a alta pressão imposta pelo uso constante da mesma molécula gerou um processo de seleção de diversos biotipos daninhos resistentes no mundo (Heap, 2017), levando o produtor a adotar novas táticas de controle, sendo uma alternativa o emprego de herbicidas com diferentes modos de ação, seja de forma isolada ou associados. Na cultura da soja, os herbicidas imazethapyr e flumioxazin têm sido

frequentemente recomendados, pois apresentam um amplo espectro de ação controlando daninhas resistentes ou de difícil controle (Norsworthy et al., 2012).

O herbicida imazethapyr pertence ao grupo químico das imidazolinonas possui como nome químico: ácido2-[4,5-dihidro-4-metil-4-(1-metiletil)-5-oxo-1H-imidazol-2-ilo]-5-etil piridinacarboxílico. A absorção pela planta acontece de forma radicular e foliar sendo que, esta última é mais rápida, onde transloca-se pelo xilema e floema, acumulando-se nos meristemas de crescimento. Pertence ao grupo de herbicidas inibidores da enzima acetolactato sintase (ALS), que é responsável pela biossíntese de três aminoácidos de cadeia ramificada valina, leucina e isoleucina (Cantwell et al., 1989; Rodrigues; Almeida, 2011). Após 48 horas da aplicação ocorre a interrupção do crescimento e dependendo da espécie, estágio de desenvolvimento e condições ambientais pode ser observado sintomas como a clorose foliar, morte do ponto de crescimento, culminando na morte total da planta.

O flumioxazin pertence ao grupo químico das ftalimidas e temcomo nome químico: 7 fluoro-6-[3,4,5,6-tetrahidro) ftalimida] 4-(2-propinil) e 1,4- benzoxa-zino-3-(2H)-one. Apresenta absorção foliar e radicular e sua translocação no simplasto é limitado. Inibidor da enzima protoporfirinogênio oxidase (PROTOX) causa em plantas sensíveis um acúmulo maciço de protoporfirinas que extravasam para o citoplasma. Neste local a ação fotossensível das protoporfirinas acumuladas provoca a peroxidação dos lipídios da membrana do citoplasma. Quando aplicados em pré-emergência das plantas daninhas provocam lesões necróticas e morte após a exposição à luz do sol. Por outro lado, em plantas tolerantes, o herbicida é rapidamente metabolizado (Rodrigues; Almeida, 2011).

Entretanto, vale ressaltar que a principal via de aplicação dos agrotóxicos é a líquida, e embora as condições de aplicação (temperatura, velocidade do vento e regulagem de equipamento) sejam adequadas, estima-se que apenas 30% do produto aplicado atinge o alvo e fica retido. Do restante, cerca de 20% não atinge a área de cultivo e 50% atinge diretamente o solo (Carneiro et al., 2015).

Uma vez no solo, parte dos compostos evapora, parte é escoada para corpos d`água superficiais, parte é lixiviada para o lençol freático e outra é degradada no ambiente terrestre (Chaim, 2004). Em decorrência desta situação, Pimentel et al., (2012) afirma que mesmo agrotóxicos com elevada eficácia, possuem baixa eficiência, sendo que alguns estudos indicam que mais de 99% dos princípios ativos aplicados são desperdiçados por não entrarem em contato com o alvo.

Além desta via de entrada, os compartimentos ambientais também recebem os produtos provenientes do escorrimento das folhas tratadas, de lavagem dos equipamentos de aplicação e liberação de resíduos de embalagens de agrotóxicos descartadas inadequadamente (Carneiro et al., 2015; Silva, 2016). Por esse motivo, diversos organismos edáficos ficam suscetíveis a diversos produtos e subprodutos. Eles podem ter seus processos vitais (ciclagem de nutrientes e energia, degradação de poluentes, estruturação do solo, decomposição da matéria orgânica, entre outros) (Van gestel, 2012), mesmo que os agrotóxicos sejam aplicados na dose comercial recomendada (Londres, 2011).

Embora o uso de herbicida seja importante para assegurar os altos índices de produtividade no sistema agrícola, há uma preocupação com o seu destino no meio ambiente e o seu impacto sobre os organismos não alvos, especificamente nos microrganismos do solo (Zabaloy et al., 2011; Van gestel, 2012). Seu uso prejudica o desempenho de funções importantes realizadas pelos microrganismos (Imfeld; Vuilleumier, 2012). O reflexo disso é a eliminação da atividade biológica do solo, que pode prejudicar a relação entre os microrganismos do solo e as culturas agrícolas que possuem uma interação direta ou indireta para promoção do crescimento vegetal.

2.3. Importância dos microganismos do solo

Os microrganismos presentes no solo, também são nomeados de microbiota, e são constituídos por: bactérias, actinomicetos, fungos, algas e protozoários. Aparte viva dos microrganismos do solo é denominada de biomassa microbiana e cerca de 90% da atividade microbiana é realizada por bactérias e fungos (Silveira; Santos, 2007).

No solo, os microrganismos realizam diversas atividades importantes (transformação da matéria orgânica, ciclagem de nutrientes e produção de compostos complexos que contribuem para agregação do solo) (Moreira; Siqueira, 2006; Mooshammer, 2014), que afetam diretamente os atributos químicos, físicos, bem como a meso e microfauna. Fato que caracteriza a importância de preservá-los, contribuindo assim para a melhoria dos sistemas agrícolas.

Alguns fatores como, disponibilidade de nutrientes, variações sazonais e distribuição de agregados podem interferir na atividade das diversas populações da comunidade microbiana do solo (Frighetto; Valarini, 2000; Hungria, 2000). Um fator relevante para o solo e para o desenvolvimento de plantas é o nitrogênio (N), onde sua disponibilidade pode ser um limitante para o sucesso das culturas (Moreira;

Siqueira, 2006).

A baixa disponibilidade de água e temperatura interfere na atividade dos microrganismos do solo, comprometendo as taxas de mineralização. Esse processo é oriundo das interações entre as condições especificas do solo, substratos distintos e diversidade dos organismos. O manejo do solo é outro fator, que também pode interferir na qualidade do solo, na quantidade de matéria orgânica, bem como, no processo de mineralização (Moreira; Siqueira 2006).

Os microrganismos presentes no solo também atuam na degradação de inúmeros compostos, como aminoácidos, proteínas, resíduos de plantas e produtos químicos, como os herbicidas (Torstensson, 1980). Segundo Childs (2007), a interação desses compostos químicos com a microbiota do solo pode gerar modificações tanto no seu tamanho, como na composição da comunidade.

Neste sentido, levando em consideração que os herbicidas estão entre os agrotóxicos mais empregados, entender o real efeito do seu uso sobre os microrganismos do solo é indispensável, uma vez que é sábio que os microrganismos do solo são responsáveis por vários processos biológicos e bioquímicos do solo.

2.4. Parâmetros utilizados na determinação de efeitos sobre os microrganismos do solo.

Para avaliar os efeitos dos contaminantes sobre a microbiota do solo, os parâmetros mais utilizados são o carbono da biomassa microbiana (Vance et al., 1987), a respiração basal do solo (evolução de CO_2) (Alef, 1995a), a atividade de enzimas do solo (Burns et al., 2013), quociente metabólico (Anderson; Domsch, 1985) e o sequenciamento de nova geração.

A quantificação do teor de C da biomassa é o parâmetro mais utilizado para determinar o impacto ambiental sobre a microbiota do solo (Gregorich,1994). Este parâmetro, pode ser indicativo do potencial da disponibilidade de nutrientes para as plantas, podendo estar relacionado à qualidade do solo e, consequentemente, à produtividade dos ecossistemas (Moreira; Siqueira, 2006). Entretanto só a determinação do CBM não fornece indicações sobre a atividade dos microrganismos do solo, sendo necessário avaliar simultaneamente parâmetros que possam medir o estado metabólico da comunidade microbiana do solo.

Segundo Gmoryová et al., (2013), a respiração basal é o parâmetro mais comumente utilizado para avaliação da atividade microbiana do solo. Este método

determina a quantidade de carbono liberado na forma de CO_2, resultante da degradação da matéria orgânica pela microbiota aeróbica do solo (EPRON, 2006). Assim como outras atividades metabólicas, esse parâmetro depende do estado fisiológico das células, sendo afetado por diversos fatores como as condições climáticas, disponibilidade de nutrientes, quantidade de C orgânico e qualidade da matéria orgânica (Alves et al., 2011; Araújo et al., 2013; Lopes et al., 2010).

A relação entre a respiração basal do solo e o CBM é denominada de quociente metabólico (qCO_2). Elevados valores de qCO_2 estão associados a ambientes submetidos a alguma condição de estresse (Jakelaitis et al., 2008; Martins et al., 2010). Por outro lado, valores baixos de qCO_2 indicam um ambiente estável ou próximo ao equilíbrio, sendo interpretado que houve um aumento da eficiência da utilização de C pela biomassa (Kaschuk; Alberton; Hungria, 2010).

A hidrólise de diacetato de fluoresceína (FDA) avalia a atividade hidrolítica total do solo. Esse substrato é hidrolisado por diferentes enzimas do solo, como as proteases, lipases e esterases, que são liberadas em grande quantidade pelos decompositores primários, servindo como indicador da atividade da biomassa do solo (Chavez et al., 2011; Melo et al., 2010). Outro parâmetro importante é a atividade da desidrogenase, que são enzimas intracelulares, produzidas por microrganismos vivos. Ela indica a capacidade oxidativa da microbiota e desempenha papel importante na degradação da matéria orgânica (Dick, 1997), sendo considerada um bom indicador da atividade microbiana (Garcia et al., 1997; Yada et al., 2015). Além disso, é capaz de indicar mudanças na atividade microbiana total em resposta às mudanças impostas ao solo (Sinha et al., 2009).

A resposta do efeito dos herbicidas sobre a estrutura e composição microbiana pode ser obtida através da metagenômica associada aos métodos de sequenciamento de alta geração (Mendes et al., 2018), que são baseados em marcadores filogenéticos como o gene 16S rRNA para bactérias.

2.5. Impacto dos herbicidas sobre os microrganismos do solo.

A Agricultura atual depende do uso de herbicidas para manejar plantas daninhas, que são uma ameaça à produtividade. Uma vez no ambiente, esses produtos químicos podem afetar os organismos do solo, (Imfeld; Vuilleumier, 2012) provocando alterações na biomassa, atividade, diversidade ou estrutura das comunidades microbianas do solo (Monkiedje et al., 2002; Girvan et al.,2004; Ros et al., 2006).

Por exemplo, Faria (2014), em estudo realizado em um solo classificado como Latossolo Vermelho Amarelo, observou que os herbicidas sulfentrazone e tebuthiuron reduziram a respiração do solo e o metilarsonato monossódico, tebuthiuron, clomazone influenciam negativamente no carbono da biomassa microbiana, onde o clomazone causou o maior impacto na atividade da microbiota. Resultado semelhante foi descrito por Zhang et al., (2010), que constataram efeitos adversos sobre a biomassa microbiana do solo, ao utilizar o imazethapyr (0,1, 1 ou 10 mgkg $^{-1}$ solo) em experimentos à campo conduzido por dois anos.

Por outro lado, Perucci e Scarponi (1994), em experimento realizado com o herbicida imazethapyr, não constataram efeito do herbicida sobre a biomassa microbiana do solo ao utilizar a dose recomendada para a cultura da soja (1,6 mg kg^{-1}).

Zilli (2008), trabalhando com Latossolo Vermelho e utilizando os herbicidas glyphosate e o imazaquim, sendo este último do mesmo grupo químico do imazethapyr, observaram que ambos os herbicidas não ocasionaram alterações significativas no teor de carbono da biomassa microbiana do solo, na respiração basal do solo e no quociente metabólico, porém, afetaram a comunidade bacteriana. De forma semelhante Moretto et al., (2017), relataram que os herbicidas atrazina, diuron e 2,4-D alteraram a composição da comunidade microbiana. Por outro lado, Dennis et al., (2018), avaliando o efeito de outros herbicida como o glifosato, glufosinato, paraquat e paraquat-diquat no solo constataram que esses herbicidas não afetaram a diversidade e estrutura microbiana do solo.

Ao se estudar o efeito dos herbicidas sobre os microrganismos do solo, observa-se uma grande variedade de resultados na literatura. Segundo Moreira; Siqueira (2006) é recorrente a dificuldade de realizar estudos onde avaliam os impactos dos pesticidas sobre a microbiota e processos biológicos do solo, pois estes são afetados por fatores relacionados a característica própria do herbicida, forma de aplicação, tipo de solo, manejo e a população de microrganismos existentes no local.

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CAPITULO I - Responses of soil microbial biomass and enzyme activity to herbicides imazethapyr and flumioxazin

Abstract

The use of herbicides is important for controlling weeds in crops. However, they can present impacts on soil properties, such as biological properties. In this study, we evaluated the responses of soil microbial biomass and enzymes activity to the application of the herbicides imazethapyr and flumioxazin and their mixture in an experiment under laboratory conditions, using soils with a different history of use. Soil microbial biomass C (MBC) decreased, while microbial biomass N (MBN) was not affected after the application of the herbicides as compared to the control. Soil respiration, respiratory quotient, and dehydrogenase (DHA) activity increased significantly after the application of the herbicides compared to the control. The hydrolysis of fluorescein diacetate (FDA) was not significantly different between the control and the herbicide treatments. The principal response curve showed the largest initial effects for the flumioxazin, followed by imazethapyr and their mixture. Flumioxazin had a different influence on soil respiration and respiratory quotient than imazethapyr and their mixture. Finally, the effects of herbicides on soil microbial biomass and enzymes are short-term as we observed recovery in the biological parameters over time.

3.1. Introduction

Te use of herbicides, as an efective practice for controlling weeds in crops, has increased in the agricultural systems in Brazil mainly due to the introduction of herbicide-resistant plants in agriculture, such as soybean and maize (Cerdeira; Duk, 2006). In Brazil, herbicides represent about 60% of the total pesticides used in agriculture (Pignati et al., 2017). Although herbicides are important for agriculture, there is a concern about their fate in the environment and their impact on soil biological properties (Zabaloy et al., 2003).

Biological properties are critically important to the ecosystem functioning since they are involved in soil organic matter decomposition, nutrient cycling, and degradation of pesticides, such as herbicides (Araujo et al., 2003). Therefore, studies assessing the effect of herbicides on soil biological properties are more important for evaluating soil quality and health (Sun et al., 2018). In addition, soil biological properties are more effective as indicators of soil quality than physical and chemical properties as they often show a faster response to an environmental impact (Nannipieri et al., 2003).

As important and responsive biological properties, soil microbial biomass and enzyme activities are frequently recommended for evaluating the effects of herbicides on the soil environment (Pose-Juan et al., 2017). Soil microbial biomass represents the active part of soil organic matter and is involved in several functions in soil, presenting a rapid turnover of soil C, N, and P; while enzymes are a suitable indicator of the catabolic activity of soil microorganism (Nannipieri et al., 2003). These biological properties are highly sensitive to detect soil disturbance after the application of chemicals, such as herbicides (Araujo et al., 2003; Pose-Juan et al., 2017; Abbas et al., 2014). For example, glyphosate, one of the most important herbicides used is soybean crops, presents a transitory and short-term effect on soil microbial biomass and activity (Nguyen et al., 2016).

Currently, glyphosate is being replaced by imazethapyr and fumioxazin in the weeds control in soybean crops, since these herbicides provide a high spectrum of action against weeds (Norsworthy et al., 2012). Imazethapyr, an herbicide belonging to the imidazoline family, acts on the grass and broadleaf weeds, being recommended for use in soybean cultivation (Rodrigues; Almeida, 2011). It has a mode of action on cell metabolism and could infuence the accumulation of microbial C (Tiour-Mauprivez et al., 2019). Flumioxazin, that belongs to the N-phenylphthalimide chemical family, is

a soil-applied herbicide recommended for broadleaf weeds control in soybean, peanut, and vineyard (Alister et al., 2008). It has a mode of action on the protoporphyrinogen oxidase (Tiour-Mauprivez et al., 2019), presenting anti-microbial effect and could inhibit some enzymes (Kushwaka; Kaushik, 2016).

Te application of imazethapyr in the soil has shown a different effect on soil microbes. A previous study conducted by Perucci and Scarponi (1994) has shown that imazethapyr, when applied at the recommended feld rate for soybean (1.6mg kg^{-1}), had no adverse efect on soil microbial biomass and activity. However, in a feld study during two years, Zhang et al., (2010). have found that the application of imazethapyr (0.1, 1 or 10mg kg^{-1} soil) changed the content of microbial biomass C. These studies have shown that would be an infuence of the history of application on soil microbial biomass and, probably, enzymes activity. On the other hand, there are no studies about the effect of fumioxazin on soil microbial biomass and enzyme activity. So far, the available studies about fumioxazin focused on its dissipation and movement in the soil, rather than its effects on microbial biomass (Alister et al., 2008; Ferrell et al., 2003).

Although the use of a mixture of herbicides seems to be more effective in controlling weeds (Norsworthy et al., 2012) it could present a complex and larger effect on non-target organisms than the individual compounds (LeBlanc; Wang, 2006). Therefore, studies evaluating the effects of the separate application of the herbicides or in a mixture on soil microbial biomass and enzyme activity are necessary for a better understanding of their efects on soil biological properties. In this context, we hypothesized that (1) the history of herbicides application and their different mode of actions could infuence the soil microbial biomass and enzyme activity; and (2) there would be a different effect of the mixture in comparison with the individual compounds. We, therefore, addressed the responses of soil microbial biomass and enzymes activity to the application of imazethapyr and flumioxazin and their mixture in a tropical soil.

3.2 Material and Methods

3.2.1 Soil sample

Soybean fields from Iria Farm, located at Sambaiba city, Maranhao, Brazil (7°31'59“S and 46°2′6″W, 243 m), which present areas with and without a previous application of the herbicides imazethapyr, flumioxazin and their mixture, were selected

for soil sampling. In the areas with previous applications of herbicides, Imazethapyr Plus NORTOX (imazethapyr), Flumyzin 500 (flumioxazin) and Zethamaxx (imazethapyr + flumioxazin) were applied, separately, during two years. These herbicides were applied in their recommended field rates which corresponded to the application of 106 g of imazethapyr ha^{-1} (1 L Imazethapyr Plus NORTOX ha^{-1} with a purity of 106 g a.i. L^{-1}), 20 g ha^{-1} of flumioxazin (40 g Flumyzin 500 ha^{-1} with a purity of 500 g a.i. kg−1) and a mixture of 127 g ha^{-1} imazethapyr and 60 g ha^{-1} flumioxazin (0.6 L Zethamaxx ha^{-1} with a purity of 212 g a.i. L^{-1} imazethapyr and 100 g a.i. L^{-1} flumioxazin).

Soil samples were collected at areas with and without a previous application of the herbicides, from the surface layer of the soil up to a depth of 20 cm and were passed through a 2-mm sieve to remove large residue fragments. Soil pH, exchangeable Ca^{2+}, Mg^{2+}, K^{+}, and the available P were estimated according to EMBRAPA (Embrapa, 1997). Total organic C (TOC) was determined by wet combustion using a mixture of 5mL of 0.167mol L^{-1} potassium dichromate and 7.5mL of concentrated sulfuric acid under heating (170 °C for 30min) (Yeomans et al., 1988). Te chemical properties of the soils are shown in Table 1.

Table 1. Chemical properties of the soils used in this study.

Soil	pH	Al^{+3}	Ca	Mg^{+2}	K^{+}	P	TOC
	$CaCl_2$		$cmol_c\ kg^{-1}$			$mgkg^{-1}$	gKg^{-1}
H0	5.0	0.8	0.92	0.66	0.3	2.83	30.72
H2	4.2	0.7	1.78	0.57	0.2	4.04	53.81

H0 - soil without history of herbicides application; H2 - soil with 2 years of herbicides application; TOC - total organic carbon.

3.2.2. Incubation experiment

Te soil samples collected at the areas with (H2) and without (H0) a previous application of the herbicides were used for the incubation experiment with the herbicides. The experiment had a completely randomized design with three replications that had the following treatments: imazethapyr (Ima); flumioxazin (Flu); flumioxazin + imazethapyr (Flu + Ima); and control without herbicide application.

The soil samples were treated, at the laboratory, with herbicides imazethapyr (Ima), flumioxazin (Flu) and their mixture (Flu + Ima). These herbicides were applied at the recommended field rates. Each soil sub-sample (1 kg; dry weight) received, respectively, 0.8 mg of imazethapyr (7.5 µL of Imazethapyr Plus NORTOX with a purity

of 106 g a.i. L^{-1}), 0.3 mg of flumioxazin (0.6 mg of Flumyzin 500 with a purity of 500 g a.i. kg^{-1}), and a mixture of 0.8 mg imazethapyr and 0.38 mg flumioxazin (3.75 µL of Zethamaxx with a purity of 212 g a.i. L^{-1} imazethapyr + 100g a.i. L^{-1} flumioxazin) that were diluted in 100mL of water and sprayed and mixed to the soils. The rates of herbicides per kg of soil were calculated considering the mass of soil in a 0–20 cm layer of a hectare. As a control, 100mL water was sprayed and mixed soil sub-samples.

Soil moisture content was adjusted to two-thirds of the field capacity and it was controlled every week through the gravimetric method. Each soil sub-samples were incubated in pots (1 kg; three replicates by treatments) in the dark for 60 days at 25 °C. Sub-samples of soil were removed from each pot for biological analysis at 0, 15, 30 and 60 days. The analysis of day 0 means that the biological data were collected immediately after the herbicide application.

3.2.3. Biological parameters

The soil respiration was monitored during aerobic incubation procedure over seven days by measuring the CO_2 evolved from 50g of soil (Alef and Nannipieri, 1995). MBC and MBN were determined in 20g soil by the chloroform fumigation-extraction method according to Vance et al., (1987) and Brookes et al., (1985), respectively. The extraction efficiency coefficients of 0.38 and 0.45 were used to convert the difference in C and N between fumigated and unfumigated soil in MBC and MBN, respectively. Moreover, we calculated the QM, as the ratio between MBC and TOC (expressed in %), and also the ratio between MBC and MBN. The respiratory quotient was calculated as CO_2-C $unit^{-1}$ microbial biomass C day^{-1}. Two grams of soil were used for estimating FDA hydrolysis according to the method of Schnurer and Rosswall (1982). DHA activity was determined using the method described in Casida et al., (1965) and based on the spectrophotometric determination of triphenyl tetrazolium formazan (TTF) released by 5 g of soil during 24 h at 35 ˚C. The data were collected at 15, 30 and 60 days. All biological analyses were conducted in triplicate and expressed as dry weight. The data were compared between treatments through the analysis of variance by ANOVA followed by post-hoc Newman-Keuls test. The means were compared by using the least signifcant difference values calculated at a 5% level.

3.2.4. Principal responses curves

The biological data were analyzed using the PRC method (Van den Brink et al.,

1999) PRC is a multivariate analysis method based on the ordination technique developed for the analysis of multivariate data sets, describing the response of communities or a set of response variables to stress in time, using a non-stressed control as a reference (Van den Brink et al., 1999). In order to make all parameters mathematically equally important in the analysis, they were standardized to zero mean and unit variance before analysis (Van den Brink et al., 1999). A separate PRC was performed for each history of previous herbicides application in field (0 and 2 years), while the statistical signifcance of the effects of the treatments was tested against the control for each incubation period using Monte Carlo permutation tests.

3.3 Results

Soil microbial biomass C (MBC) decreased signifcantly afer the application of the herbicides as compared to the control in both areas with (H2) and without (H0) a previous application of the herbicides (Fig. 1A), while microbial biomass N (MBN) was not signifcantly different between the control and the herbicide treatments (Fig. 1B). During the incubation, MBC decreased at 15 days after herbicides application and increased at 30 and 60 days. In contrast, MBC increased from 0 to 60 days in the control. On the other hand, MBN did not decrease signifcantly from 0 to 60 da

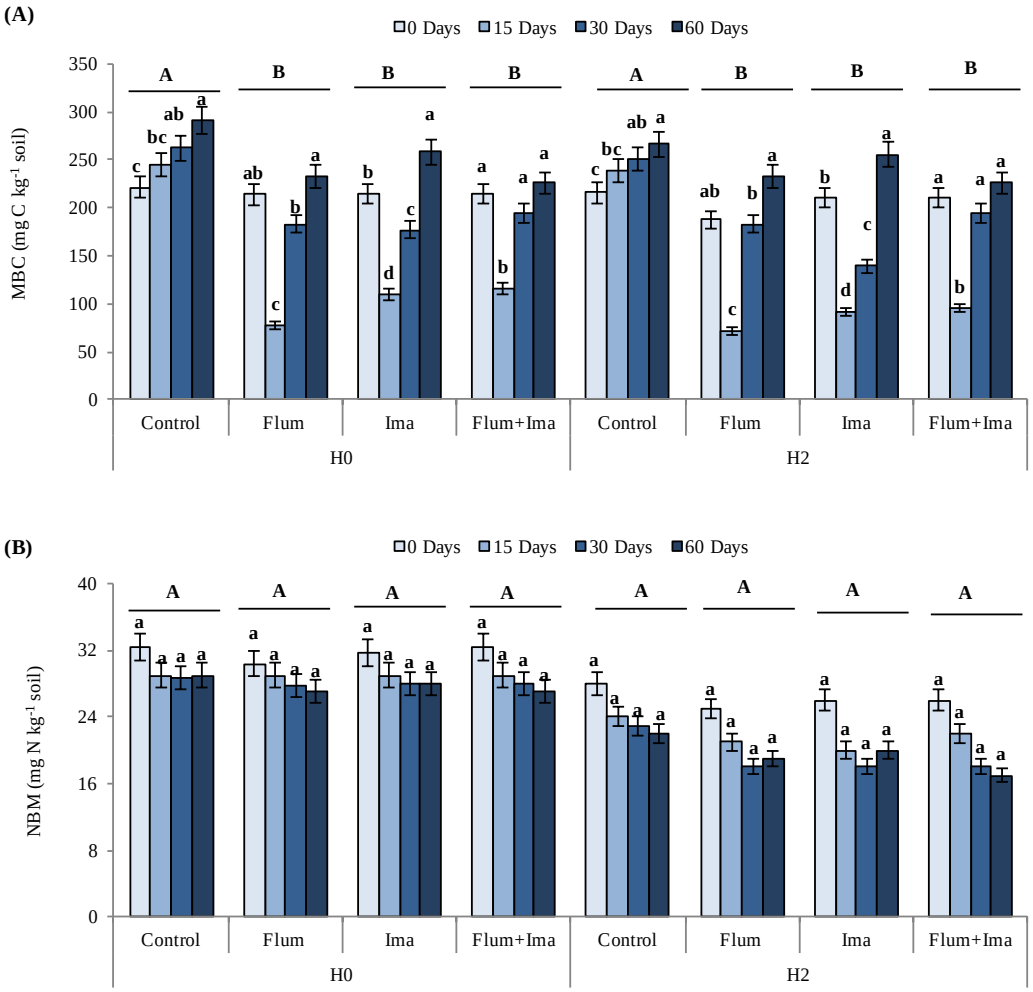

Figure 1. Microbial biomass C **(A)** and N **(B)** in soils, without (H0) and with (H2) history of herbicides application in the feld, untreated (control) and treated with Flumioxazin (Flum), imazethapyr (Ima) and their mixture (Flum+Ima), at diferent incubation times. Bars represent the SD of the mean. Te diferent lower-case letters above the bars indicate signifcant differences (P<0.05) between sampling times for each treatment and different upper-case letters above the bars indicate significant differences (P<0.005) between treatments (mean values) for each soil.

The microbial quotient (QM) did not show differences between the control and the herbicides treatments (Fig. 2A), while MBC:MBN ratio decreased after the application of the herbicides as compared to the control in both H0 and H2 (Fig. 2B). During the incubation, QM, and MBC:MBN ratio decreased at 15 days after herbicides application and increased at 30 and 60 days (Fig. 2).

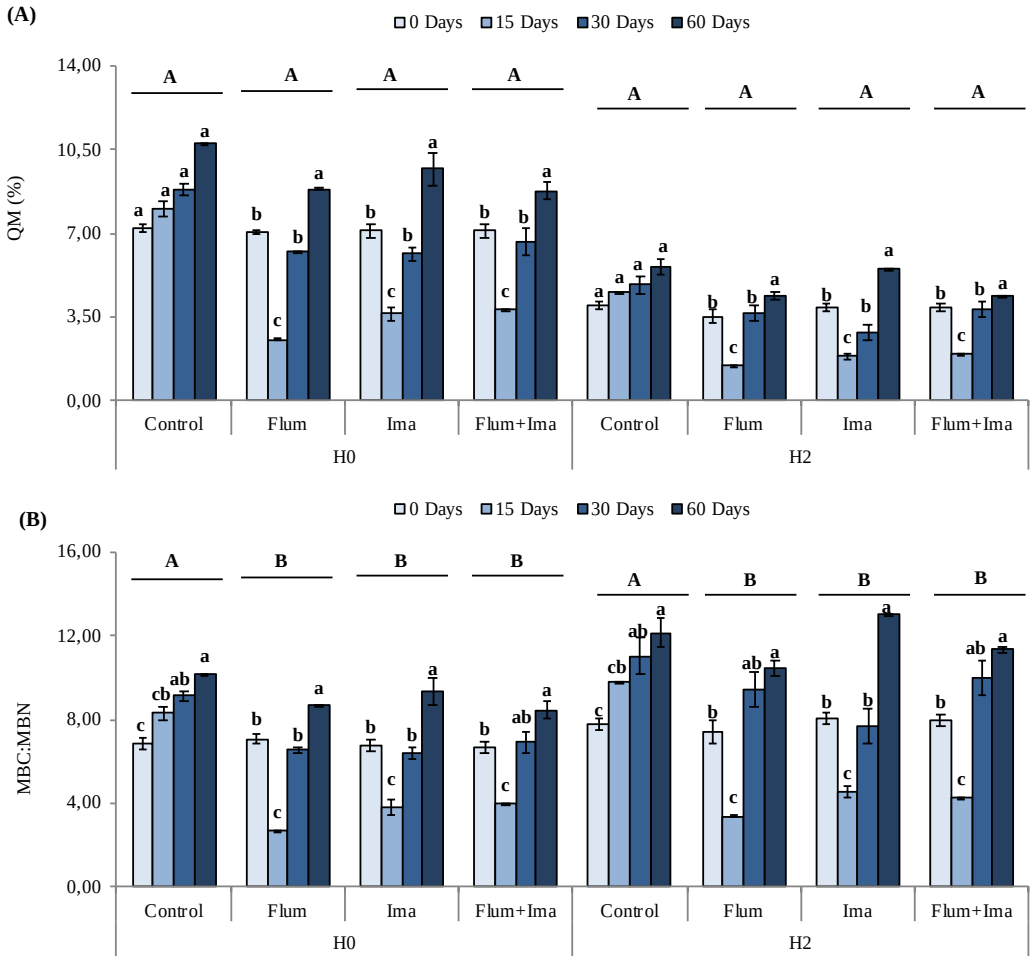

Figure 2. Microbial quotient (QM) **(A)** and MBC:MBN ratio **(B)** in soils, without (H0) and with (H2) history of herbicides application in the feld, untreated (control) and treated with Flumioxazin (Flum), imazethapyr (Ima) and their mixture (Flum+Ima), at diferent incubation times. Bars represent the SD of the mean. Te diferent lower-case letters above the bars indicate signifcant diferences (P<0.05) between sampling times for each treatment and diferent upper-case letters above the bars indicate signifcant diferences (P<0.05) between treatments (mean values) for each soil.

Soil respiration (Fig. 3A) and respiratory quotient (Fig. 3B) increased signifcantly after the application of the herbicides as compared to the control in both H0 and H2 soils. During the incubation, soil respiration and respiratory quotient increased, in both H0 and H2, at 15 days after herbicides application and decreased at 30 and 60 days. Interestingly, soil respiration decreases and respiratory quotient did not vary during the period of incubation in the control (Fig. 3).

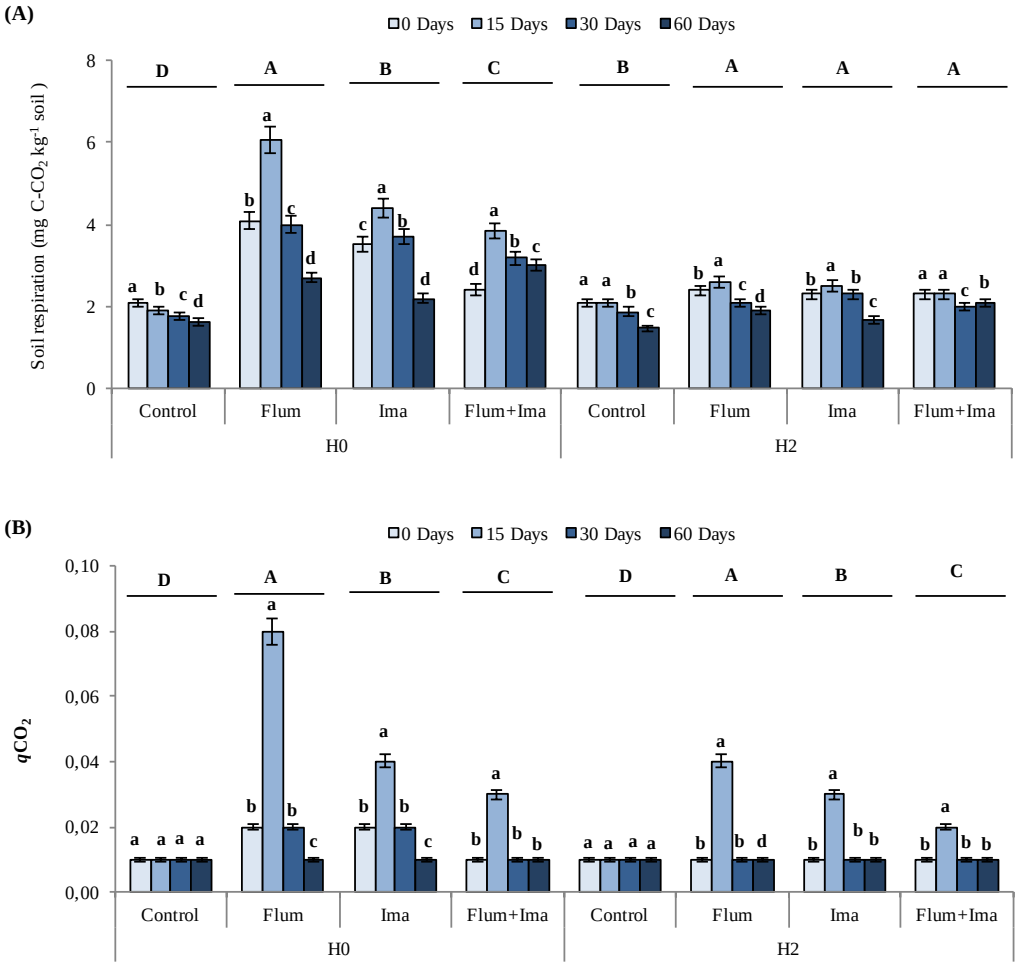

Figure 3. Soil respiration **(A)** and respiratory quotient **(B)** in soils, without (H0) and with (H2) history of herbicides application in the feld, untreated (control) and treated with Flumioxazin (Flum), imazethapyr (Ima) and their mixture (Flum+Ima), at diferent incubation times. Bars represent the SD of the mean. Te diferent lower-case letters above the bars indicate signifcant diferences (P<0.05) between sampling times for each treatment and different upper-case letters above the bars indicate signifcant diferences (P<0.05) between treatments (mean values) for each soil.

Except for flumioxazin in H0 soil, dehydrogenase activity (DHA) increased signifcantly after the application of the herbicides as compared to the control in both H0 and H2 soils (Fig. 4A), while the hydrolysis of fluorescein diacetate (FDA) was not signifcantly different between the control and the herbicide treatments (Fig. 4B). During the incubation, DHA increased in both H0 and H2 at 15 days after herbicides application and decreased at 30 and 60 days. In contrast, FDA decreased in both H0 and H2 at 15 days after herbicides application and increased at 30 and 60 days.

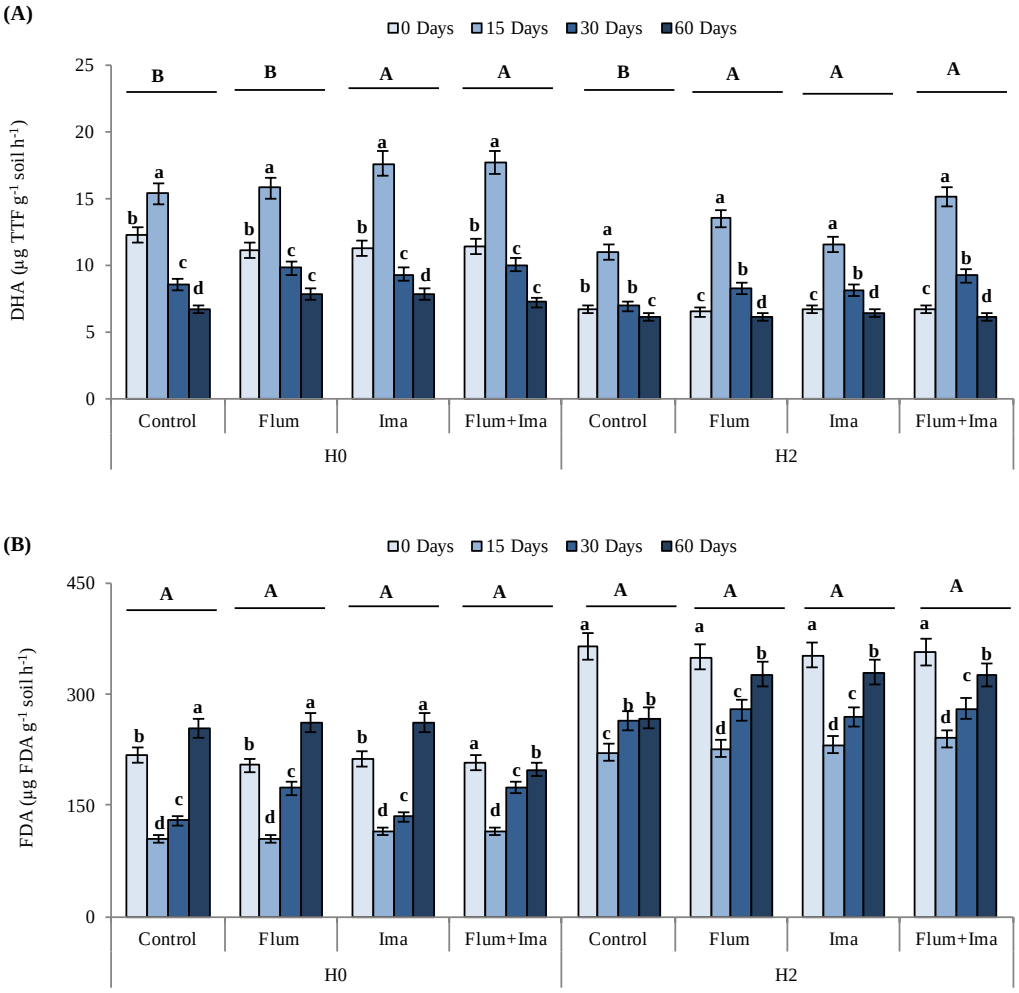

Figure 4. Dehydrogenase activity **(A)** and fuorescein diacetate hydrolysis **(B)** in soils, without (H0) and with (H2) history of herbicides application in the feld, untreated (control) and treated with Flumioxazin (Flum), imazethapyr (Ima) and their mixture (Flum+Ima), at diferent incubation times. Bars represent the SD of the mean. Te diferent lower-case letters above the bars indicate signifcant diferences (P<0.05) between sampling times for each treatment and diferent upper-case letters above the bars indicate signifcant diferences (P<0.05) between treatments (mean values) for each soil

The Principal Response Curves (PRC) analysis showed that a signifcant part of the treatment variation was displayed in both H0 and H2 PRC diagrams (Fig. 5). The H0 soil samples showed the largest initial effects for the flumioxazin, followed by imazethapyr and their mixture (Fig. 5A). Partial recovery was also indicated. MBC, QM, and the MBC:MBN ratio decreased in values due to the treatments, while the respiratory quotient and soil respiration increased. MBN, DHA, and FDA showed no response. The H2 soil samples showed equal effect sizes for the three treatments and a full recovery for the imazethapyr and partial recovery for the flumioxazin and their

mixture (Fig. 5B). MBC, MBN, QM, and the MBC:MBN ratio decreased in values; respiratory quotient, soil respiration, and DHA increased while FDA showed no response. For both the H0 and H2 soils, the treatments had a signifcant effect on the biological parameters at all periods of incubation. Te individual treatments could not be tested against the control due to a limited number of permutation possibilities.

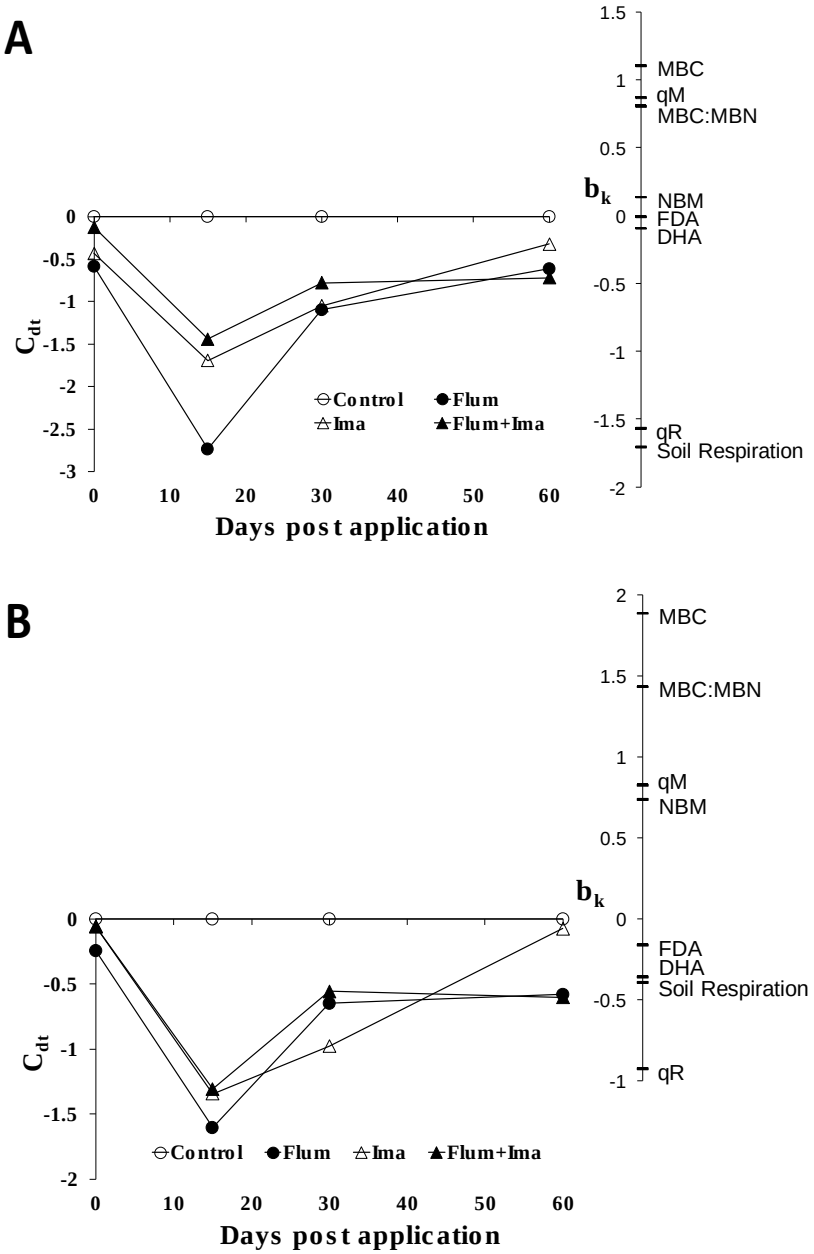

Figure 5. PRC diagrams showing the response of the biological parameters to the herbicide treatments for H0 **(A)** and H2 **(B)** during the incubation time. Incubation time explained 58 and 67% of the total variation in the biological parameter values of the H0 and H2 data sets, respectively. Treatment explained 41 and 31% of the H0 and H2 data sets, of which 88 and 87% are displayed in the respective PRC diagrams.

3.4. Discussion

In this study, we assessed the effect of the herbicides imazethapyr, flumioxazin, and their mixture on soil microbial biomass and enzyme activity in soil with (H2) and without (H0) previous application of these herbicides in the field. Partially, in contrast with the first hypothesis, we did not find the infuence of the previous application of herbicides on soil microbial biomass and respiration (Figs. 1 and 3). However, the enzyme activity was infuenced by the previous application of herbicides (Fig. 4).

According to Moorman (1994), the cumulative effect of repeated annual applications of herbicides may, in some cases, not infuence soil biological properties and it may be related to degradation time (DT) of the compound in the soil. Although in this study we did not evaluate DT of these herbicides in the soil, the available data have reported that the DTs of imazethapyr and flumioxazin, in similar soil types, ranges from 7-19 and 15-20 days, respectively (PPDB, 2019). Therefore, in our soils, both herbicides seem to be non-persistent and would not have an influence on the responses of soil biological parameters after two years.

MBC was influenced by herbicides application in both H0 and H2 as compared to the control, while MBN was not influenced by the herbicides (Fig. 1). Interestingly, MBC decreased due to the herbicides applications while MBN was not affected. It suggests that these herbicides do not have a toxic effect on microbial N, which agrees with Sawicka and Selwet (2006), who reported that imazethapyr applied at 90g ha^{-1} does not present a negative efect on N related microorganisms. In this study, we have applied imazethapyr at 106g ha^{-1}, which is closer to the rate used by Sawicka and Selwet (1998). For flumioxazin, there is no study about this herbicide on soil microbial biomass N.

During the incubation, MBC decreased in the first 15 days after herbicides application (Fig. 1A). It agrees with Zhang et al., (2010) who observed that MBC decreased during the initial incubation period after application of imazethapyr at 0.1, 1 and 10mg kg^{-1} soil. There are no previous studies on the effect of flumioxazin on MBC and, therefore, our results suggest that flumioxazin, initially, had a negative effect on soil MBC. The results also show that after the initial negative effect, MBC recovered and increased until the end of the incubation period. This recovery of the soil microbial biomass C may be related to the degradation of these herbicides and their decreasing toxicity for soil microbial biomass (Perucci; Scarponi, 1994). Also, the initial lyses of microbial cells promoted by herbicides could have increased the content of C and,

thus, contribute to C and energy sources for soil microbial biomass.

The MBC:MBN ratio was influenced by herbicides application in both H0 and H2 as compared to the control, while the microbial quotient (QM) was not influenced by the herbicides (Fig. 2). During the incubation, both parameters decreased during the first 15 days after the herbicide application and recovered to control values after this initial period. This response is similar to the one observed for MBC and indicates that any effect of herbicides on microbial biomass C influences the status of these indices.

Soil respiration is considered an indicator of microbial activity (Alef; Nannipieri, 1995), while respiratory quotient is a useful indicator of ecological disorder or disturbance in soil (Melo et al., 2017). The results showed that soil respiration and respiratory quotient increased after the application of herbicides in both H0 and H2 as compared to the control (Fig. 3). Although the increase in soil respiration could suggest a positive effect of the herbicides on microbial activity, the increase in respiratory quotient indicates an initial negative effect of the herbicides on soil microorganisms. During the incubation, soil respiration and respiratory quotient increased during the first 15 days. It occurs since the application of chemical compounds in soil requires an adaptation of soil microbial biomass that uses their reserves to degrade these compounds. Thus, C from microbial biomass is lost, increasing the respiratory quotient. After 15 days, these parameters decreased back to control levels and this pattern could suggest an adaptation of the remaining microbial biomass that increased between 15 to 60 days.

DHA did not vary between the treatments and the control with the application of flumioxazin in the H0 soil, suggesting no effect of flumioxazin on catabolic activity in soil (Jarvan et al., 2014). However, DHA was affected by herbicides in H2 soil (Fig. 4A). After the incubation, DHA increased during the first 15 days and decreased after that period to control levels. This pattern was similar to the one observed for soil respiration (Fig. 3A) and it can indicate an initial stimulation of microbial activity by the herbicides. Interestingly, the FDA was not infuenced by herbicides application in both H0 and H2 as compared to the control (Fig. 4B) suggesting no detrimental effect on soil microbial activity.

Finally, we show the pattern of these biological properties in response to herbicides using the multivariate method of PRC. PRC showed signifcant positive and negative effects of herbicides on soil biological parameters (Fig. 5). Interestingly, the

herbicide flumioxazin affected negatively the MBC and their correlated parameters, i.e. microbial quotient and MBC:MBN ratio, in the soil with no history of herbicide application (Figs. 1A, 2 and 5A). It suggests that the C-related microorganisms did not adapt to this herbicide even being applied for two years. In contrast, the parameter related to soil disturbance, i.e. respiratory quotient, increased as a response to the possible stress caused by the herbicides to soil microbial biomass (Figs. 3B and 5). When we consider the soil with previous application of the herbicides, separated and in a mixture, they present the same effect on biological parameters with negative and positive responses to microbial biomass and respiration, respectively (Fig. 5B). However, the biological parameters present a full recovery in the treatment with the application of imazethapyr, which suggests that the microbial biomass can be more adapted to this herbicide than to flumioxazin. Previously, Perucci and Scarponi (1994) have also found a recovery of microbial properties after the application of imazethapyr when it was applied at the recommended feld rate.

3.4.1. Ecological implications

The results of this study show that the evaluated parameters increased, decreased or did not show response during the first 15 days to the application of herbicides. MBC, QM, MBC:MBN ratio and FDA decreased initially after the application of herbicides. On the other hand, soil respiration, respiratory quotient, and DHA increased as a direct effect of herbicides. These responses can be related to the mode of action of the herbicides. The herbicide imazethapyr belongs to the class of imidazolinones and has a mode of action on cell metabolism, inhibiting acetohydroxyacid synthase (AHAS) and regulating the biosynthesis of some compounds (Rossa; Schloss, 1984) Thiour-Mauprivez et al., (2019) reported AHAS influencing on the accumulation of microbial C and the microbial activity, such as FDA hydrolysis. Also, since AHAS disrupts the accumulation of microbial C, this element can be lost through catabolic respiration, i.e. increasing respiratory quotient and dehydrogenase.

Regarding flumioxazin, it belongs to the N-phenylphthalimide chemical family and has a protoporphyrinogen oxidase mode of action (Thiour-Mauprivez et al., (2019). So far, no ecotoxicological studies were done with flumioxazin. Although N-phenylphthalimide chemicals have an anti-microbial effect and inhibit some enzymes (Kushwaka; Kaushik, 2016) this compound can protect N from its catabolisms

(Hassanzadeh et al., 2007). Thus, although flumioxazin decreases temporally the microbial biomass C, it may have protected microbial biomass N, so presenting a neutral effect (Fig. 1).

The responses of soil biological parameters to herbicides can indicate some ecological implications for soil quality. The application of herbicides could decrease temporally the C pool, microbial stoichiometry, and activity. This result indicates a C limitation in soil and also a decrease in the organic matter decomposition by microbes (Nicolás et al., 2019). On the other hand, herbicides promoted losses of C, by catabolic respiration, as an indication of microbial disturbance (Melo et al., 2017). Interestingly, these herbicides did not present an effect on N storage in soil and potential N cycling (Van et al., 2015). Finally, both herbicides are degraded rapidly in the soil through microbial degradation. Thus, it could explain this temporary effect of these herbicides on soil biological parameters.

3.5. Conclusion

In this study, the application of herbicides infuenced the soil microbial biomass and enzyme activity differently. In general, the application of the herbicides influenced the soil microbial biomass C, while the hydrolysis of the FDA was not affected. Flumioxazin had a different influence on soil respiration and respiratory quotient than imazethapyr and their mixture. The mixture did not present different effects on soil microbial biomass and enzyme activity than the individual compounds. Finally, the effects of herbicides on soil microbial biomass and enzymes are short-term as we observed a recovery in the biological parameters over time.

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CAPITULO II – Response of soil bacterial communities to the application of the herbicides imazethapyr and flumyzin

Abstract

The herbicides imazethapyr and flumyzin have been recommended for controlling weeds, mainly in soybean fields. However, it is unclear the effect of these herbicides on soil microbial communities. In this study, we assessed the responses of bacterial community to the application of imazethapyr and flumyzin in soil. Soil samples were incubated with the application of the herbicides imazethapyr, flumyzin, and their mixture, and analyzed at 0, 15, 30, and 60 days. The abundance of specific microbial phyla changed as a response of the herbicide application with a significant decrease of Acidobacteria, Verrucomicrobia, Elusimicrobia, and Planctomycetes, and an increase of Gemmatimonadetes, Bacteroidetes, Firmicutes, and Proteobacteria. The principal component analysis clustered the samples according to the time of incubation and the microbial diversity increased on the 15th day with a trend to decrease at 30 and 60 days. The evaluation of potential functions has shown the core functions represented by chemoheterotrophy (35.1%), followed by aerobic chemoheterotrophy (32.8%), nitrification (6.2%), ammonia oxidation (6.2%), and aromatic compound degradation (4.4%). Our data showed an increase of microbial groups that have the potential to metabolize the chemical compounds, such as the genera *Bryobacter, Gaiella, and Flavobacterium,* which increased significantly in abundance and can be explored for future biotechnological use. Finally, this study shows that the application of the herbicides in soil affects the microbial profile with the potential to affect functions mediated by microbial communities, and this effect is related to groups with the potential to degrade the compound.

Keywords: Soil microbiome; next-generation sequencing; functional prediction; soil microbial ecology.

4.1. Introduction

The introduction of herbicide-resistant crops in Brazilian agriculture has promoted an increase in the utilization of herbicides to control weeds in several crops (Cerdeira; Duke, 2016). Nowadays, the application of herbicides in Brazil accounts for about 60% of the total pesticides (Pignati et al., 2017) and, especially to soybean, the herbicides represent an important input to increase the crop yield.

In soybean management, the herbicides imazethapyr and flumyzin have been recommended for controlling weeds as these herbicides increase the spectrum of action (Norsworthy et al., 2012). The imazethapyr belongs to the imidazoline family and it is used as a selective herbicide applied at pre- and post-emergence against grasses and broadleaf weeds (Rodrigues; Almeida, 2012); while the flumyzin is a pre-emergent soil-applied herbicide used for weeds control in soybean and peanut (Alister et al., 2008). Although these herbicides have been used massively in agriculture, little information about their effect on the soil environment is available. Previous studies have reported that imazethapyr presents different impacts on soil microbial biomass (Perucci; Scarponi, 1994; Zang et al., 2010). For example, Zhang et al., (2010) reported that imazethapyr applied at several rates (0.1, 1, or 10 mg kg^{-1} soil) affected soil microbial biomass, although Perucci and Scarponi (1994) did not find any effect of imazethapyr on microbial biomass at field rate. In addition, there are some reports about its persistence and movement in the soil (Alister et al., 2008; Ferrel; Vencill, 2003), but, so far, there are no studies about the effect of flumyzin on soil microorganisms.

As reported above, the studies about imazethapyr have focused on soil microbial biomass, while there is no information about flumyzin and its effect on soil microorganisms. Thus, it remains unclear the effect of imazethapyr and flumyzin on soil microbial communities, especially bacterial communities. Soil bacteria act on several soil functions, such as organic matter decomposition and nutrient cycling (Miranda et al., 2018), being important in carbon (C) and nitrogen (N) turnover in the terrestrial environment (Bengtson; Sterngren; Rousk, 2012). Therefore, the diversity and structure of the bacterial community are important for soil functioning (Hansel et al., 2008). In this sense, the application of herbicides can affect the microbial community and, consequently, influence the soil functioning. Indeed, recent studies have assessed the effect of herbicides application on soil bacterial microbial community and reported negative (Morreto et al., 2017), positive (Crouzet et al., 2010),

and neutral (Dennis et al., 2019) responses of microbial groups to different herbicides. Dennis et al., (2019) assessed the effect of glyphosate, glufosinate, paraquat, and paraquat-diquat on soil and found that these herbicides did not affect the microbial diversity and structure in the soil. On the other hand, Moretto et al., (2017) reported that atrazine, diuron, and 2,4-D changed the composition of the microbial community, being atrazine and diuron the most effective on microbial groups.

Considering that there is no information about the effect of imazethapyr and flumyzin, in isolate application or in a mixture, on soil microorganisms, this study aimed to address the response of the bacterial community to the application of these herbicides in soil. We hypothesize that different herbicides could present different effects on the diversity, structure, and composition of the microbial community in soil when applied separated or combined. To test this hypothesis, we assessed the bacterial community structure and composition through the sequencing of the 16S rRNA gene.

4.2. Material and methodos

4.2.1 Soil sample

Soil sampling was performed at a commercial soybean field from Iria Farm, located at Sambaiba city, Maranhão, Brazil (7°31'59''S and 46°2'6''W, 243 m), where the soil was never treated with the herbicides imazethapyr, flumyzin, and their mixture.

The area was subdivided into three similar plots (400 m^2 each). In each plot, five subsamples were collected, at 0-20 cm depth, and pooled together to form a composite sample. All samples were put into plastic bags and transported under low temperature to the laboratory. They were kept in a refrigerator at 4-8°C, being processed and analyzed two weeks later. The samples were passed through a 2-mm sieve to remove large residue fragments. Soil pH, exchangeable Ca^{2+}, Mg^{2+}, K^{+}, and the available P were estimated according to EMBRAPA (1997). Total organic C (TOC) was determined by wet combustion using a mixture of 5 mL of 0.167 mol L^{-1} potassium dichromate and 7.5 mL of concentrated sulfuric acid under heating (170 ºC for 30 min) (Yeomans; Bremner, 1988). The chemical properties of the soils are shown in Table 1.

4.2.2 Incubacion experiment

The soil samples were incubated with different herbicides treatments consisting of imazethapyr (Ima), flumyzin (Flu), and their mixture (Flu+Ima), in three replications,

applied at the recommended field rates. Before the application of herbicides, the gravimetric soil moisture of each soil was estimated in order to apply the correct herbicides rates. Thus, each soil sample (1 kg; dry weight) received, respectively, 0.8 mg of imazethapyr (7.5 µL of Imazethapyr Plus Nortox® with a purity of 106 g a.i. L^{-1}), 0.3 mg of flumyzin (0.6 mg of Flumyzin 500® with a purity of 500 g a.i. kg^{-1}), and a mixture of 0.8 mg imazethapyr and 0.38 mg flumyzin (3.75 µL of Zethamaxx® with a purity of 212 g a.i. L^{-1} imazethapyr + 100 g a.i. L^{-1} flumyzin) that were diluted in 100 mL of water and sprayed and mixed to the soils. As a control, 100 mL water was sprayed and mixed in soil sub-samples.

Soil moisture content was adjusted to two-thirds of the field capacity for each soil sample according to their initial moisture. Soil samples were incubated in jars in the dark for 60 days at 25 °C. Sub-samples of soil were removed from each jar analysis at 0, 15, 30, and 60 days. The 0 days analysis means that the microbial data were collected immediately after herbicide application. In each period, a portion of 0.5 g of soil was collected from the soil sub-samples inside the jars.

4.2.3 DNA extraction, sequencing, and processing

Total DNA was extracted from 0.5g (total humid weight) of soil using the PowerLyzer PowerSoil DNA Isolation Kit (MoBIO Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions.

The V4 region of the 16S rRNA gene was amplified with region-specific primers (515F/806R) (Caporaso et al., 2011) Each 25 µl Polymerase Chain Reaction (PCR) reaction volume contained the following: 12.25µL of nuclease-free water (Certified Nuclease-free, Promega, Madison, WI, USA), 5.0µL of buffer solution 5x (MgCl2 2Mm), 0.3mM dNTP's 0.3µM of each primer (515 YF 40µM e 806 R 10 µM), 1.0 unit of Platinum Taq polymerase High Fidelity in a concentration of 0.5 µL (Invitrogen, Carlsbad, CA, USA), and 2.0 µL of template DNA previously diluted at 10ng μL^{-1}. Moreover, a control reaction was performed by adding water in place of DNA. The conditions for PCR were as follows: 95°C for 3 min to denature the DNA, with 35 cycles at 98°C for 20s, 55°C for 20 s, and 72°C for 30s, with a final extension of 3 min at 72°C to ensure complete elongation.

After indexing, the PCR products were cleaned up using Agencourt AMPure XP – PCR purification beads (Beckman Coulter, Brea, CA, USA), according to the

manufacturer's manual, and quantified using the dsDNA BR assay Kit (Invitrogen, Carlsbad, CA, USA) on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Once quantified, an equimolar concentration of each library (50 ng) was pooled into a single tube. After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured, and then diluted to a final concentration of 8.0 pM with a 20% PhiX (Illumina, San Diego, CA, USA). Diluted pooled samples were loaded on an Illumina MiSeq (Illumina, San Diego, CA, USA) and sequenced using a 500-cycle (paired-end sequencing 2x250).

The 16S rRNA gene sequencing data were processed using QIIME 2 version 2017.11 (Bolyen, M; Rideout, J.R; Dillon, M.r, 2019). Firstly, the sequences were demultiplexed and the quality control was carried out using DADA2 (Callahan et al., 2016) using the consensus method to remove any remaining chimeric and low-quality sequences. After filtering, approximately 3 million high-quality reads (on average, ~ 68,000 reads per sample) were obtained. The samples were rarefied to 21,000 sequences per sample, following the number of the lowest sample, and singletons and doubletons were removed. For taxonomic assignment, OTU centroid sequences were queried against the Silva database v.132 (Quast et al., 2013) at 97% similarity using a trained Naïve Bayes classifier implemented in QIIME 2. The generated matrix was further used for statistical analyses. To further assess the relevant potential functional profile of the bacterial community, we performed a functional annotation using the FAPROTAX database (Louca; Parfrey; Doebeli, 2016). For this, a table of frequency of taxa at the genus level was used as input and converted into a putative functional table. The 16S rRNA gene sequences were deposited at the NCBI under the accession number PRJNA607071.

4.2.4 Statistical analysis

A principal component analysis (PCA) was performed to compare the microbial community structure among the treatments using Canoco 4.5 (Biometrics, Wageningen, The Netherlands). For this, the data was first evaluated using Detrended correspondence analysis (DCA) to test data normality and indicated that the best-fit mathematical model found was linear, indicating the use of PCA. Additionally, the permutation multivariate analysis of variance based on Bray-Curtis (PERMANOVA; (Anderson, 2001)) was applied to test whether the treatments harbored significantly different bacterial community composition. Shannon diversity index was calculated

based on the OTU table. Diversity measurements and PERMANOVA were calculated using the Past v.3 (Hammer; Harper; Ryan, 2001). To assess variation in the relative abundance of the bacterial community, the OTU table was used as input in the software STAMP (Parks; Beiko, 2010). The *P*-values were calculated based on two-sided Welch's t-test and the correction was made using Benjamini-Hochberg FDR. For visualization of the community composition, heatmaps were constructed based on z-score transformed phylum abundance.

4.3. Results

4.3.1 Bacterial community structure, diversity, and composition

The 16S rRNA sequencing generated approximately 2,980,00 sequences. After quality trimming and rarefaction (Supplementary Figure S1), the sequences were clustered into approximately 8,500 OTUs. In general, the samples were dominated by Proteobacteria (22.6% of the sequences), followed by Actinobacteria (20.6%), Firmicutes (8.7%), Planctomycetes (6.6%), Acidobacteria (5.3%), Chloroflexi (2.5%), Verrucomicrobia (1.9%), and Bacteroidetes (1.4%). A total of 28.22% belonged to unclassified bacteria. The relative abundance of specific microbial phyla changed as a response of the herbicide application with a significant decrease of Acidobacteria, Verrucomicrobia, Elusimicrobia, and Planctomycetes, and an increase of Gemmatimonadetes, Bacteroidetes, Firmicutes, and Proteobacteria (Supplementary Figure S2).

The two first axes of the principal component analysis (PCA) display 69% of the data variation in the bacterial community composition. Samples were according to the time after the herbicide application (Figure 6; PERMANOVA F=17.37, *P*=0.0001). In a comparison at the OTU level, no differences were found between the treatments (F= 1.55, *P*=0.0845; Interaction F=0.85, *P*=0.72). The Shannon index showed an increase in microbial diversity after the herbicide application on the 15th day, followed by a trend to decrease at 30 and 60 days (Figure 7).

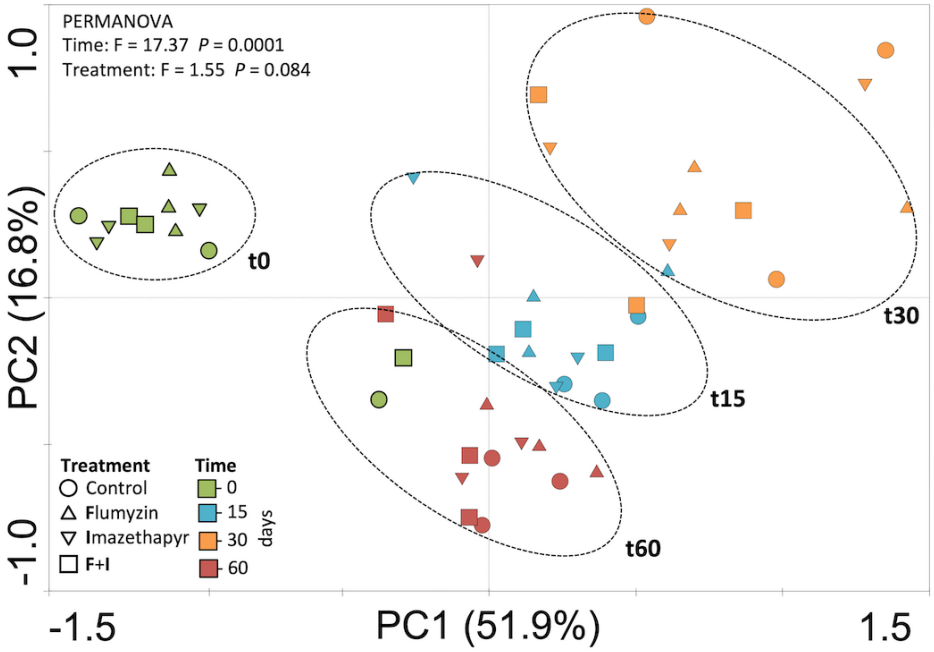

Figure 6. Principal component analysis (PCA) of bacterial community structure patterns in soils with the application of herbicides during 60 days. The taxonomic bacterial and archaeal profiling was based on 16S rRNA OTUs classified at 97% of similarity. The dashed lines indicate significant clusters by permutation analysis based on time factor (PERMANOVA, P < 0.05).

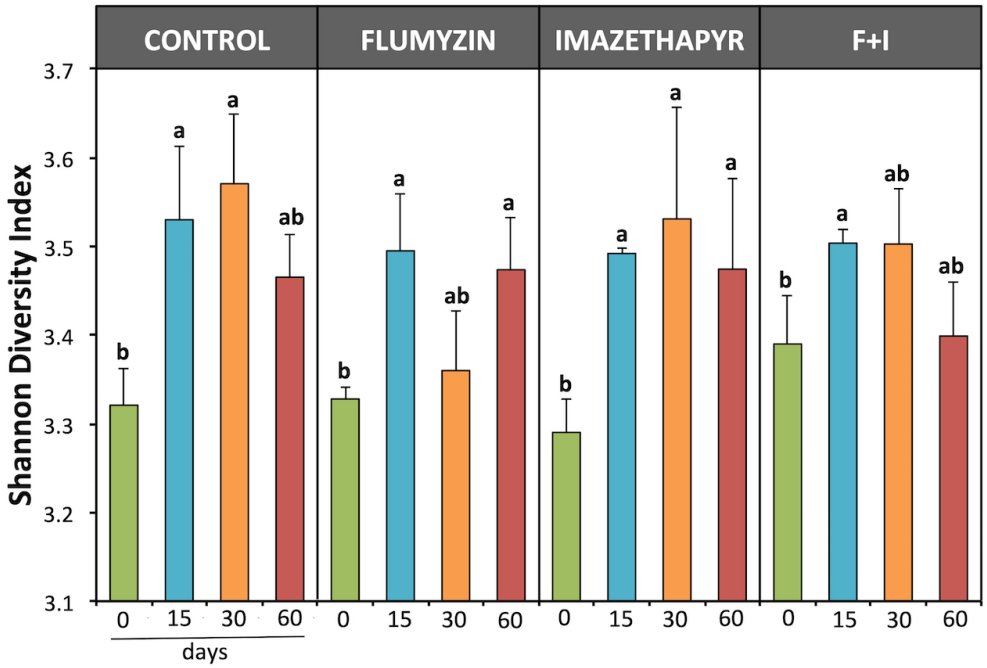

Figure 7. Effect of herbicide application on the diversity of soil bacterial communities during 60 days. Taxonomic diversity based on 16S rRNA OTUs classified at 97% of similarity. Different lower-case letters indicate significant differences based on the Tukey test (P < 0.05).

Further analysis at the genus level showed that 15 genera were affected by flumyzin application, while seven and eight were affected by the application of imazethapyr and the combination of both, respectively (Figure 8). In the treatment with flumyzin, there was a high abundance of the genus *Bryobacter* and members of Acidimicrobiia and Acidobacteriales (Figure 8A). In the soil treated with imazethapyr, the genus *Gaiella* showed the highest abundance (Figure 8B), while in the treatment with the application of the two herbicides in combination, the genus *Conexibacterium* was the most abundant (Figure 8C). Interestingly, the genus *Gaiella* and a genus from the family Fimbriimonadaceae increased in abundance in all treatments with the application of the herbicides.

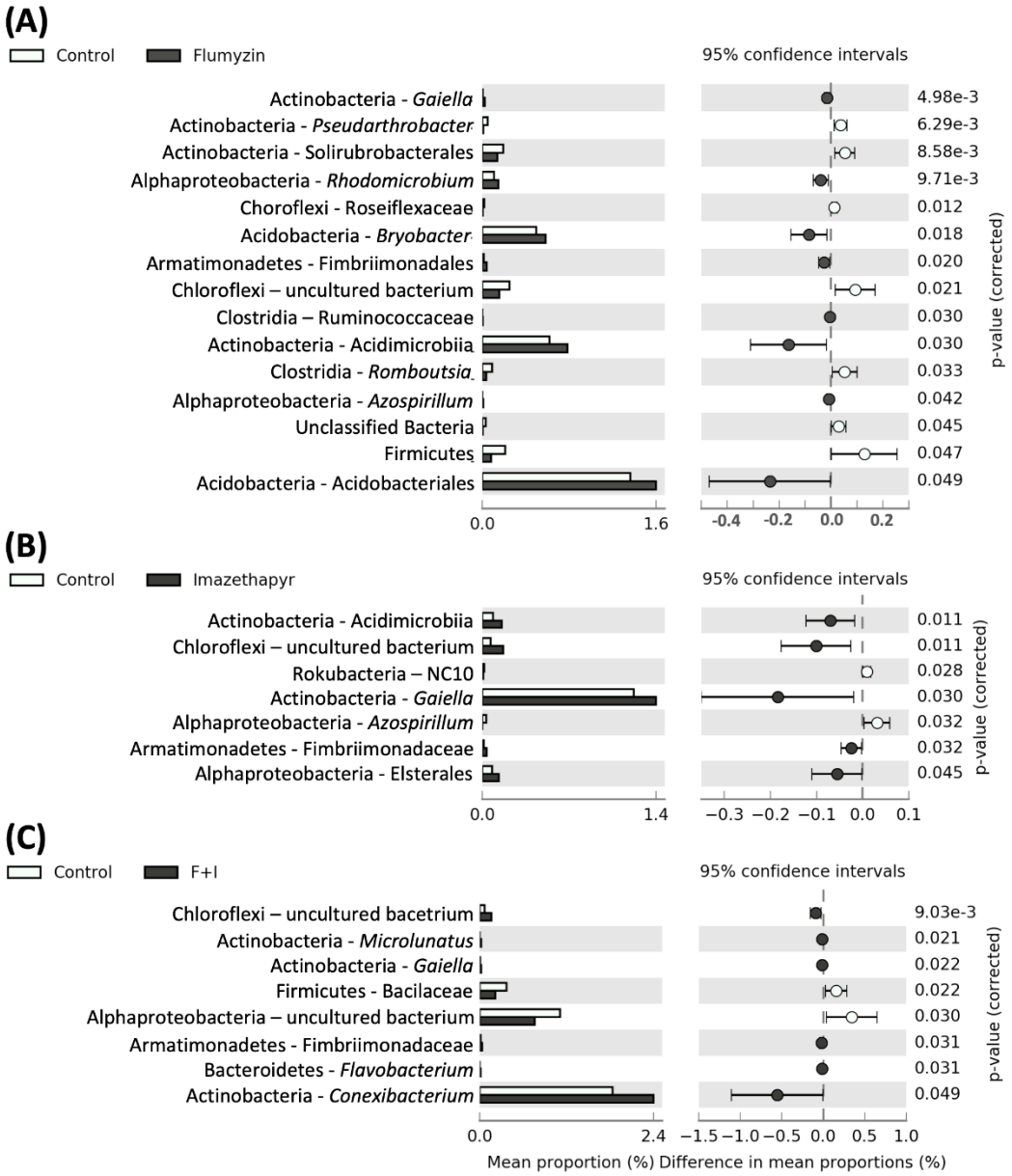

Figure 8. Plots showing the genera with differential abundance in soils with herbicide application comparing the control with the application of (A) Flumyzin, (B) Imazethapyr, and (C) F+I. *P*-values were calculated based on Welch's t-test with Benjamini-Hochberg correction ($P < 0.05$).

4.3.2 Functional prediction of the bacterial community

To assess the potential functions of the soil bacterial community and evaluate the effects of the application of the herbicides on these functions, the OTUs were classified into 38 functional groups, with the core functions represented by chemoheterotrophy (35.1%), followed by aerobic chemoheterotrophy (32.8%), nitrification (6.2%), ammonia oxidation (6.2%), and aromatic compound degradation (4.4%). We observed that several potential functions were significantly altered by the

herbicide application (Figure 9).

In the treatment with flumyzin, there was a decrease of microbial groups related to chemoheterotrophy and an increase related to animal parasites or symbiont; also, there was an increase of the potential function related to ureolysis (Figure 9B). For the imazethapyr, we observed an increase of sequences related to hydrogen oxidation, reductive acetogenesis, and ureolysis 60 days after the herbicide application (Figure 9C). When the application of the herbicides was in combination, there was an increase of animal parasites or symbionts, ureolysis, and hydrocarbon degradation (Figure 9D). In addition, we compared the effect of the application of the herbicides with the control soils (Figure 10). The application of the flumyzin increased the abundance of groups with the potential of chemoheterotrophy, and the application of the two herbicides together increased the groups related to xylanolysis and the abundance of plant pathogens.

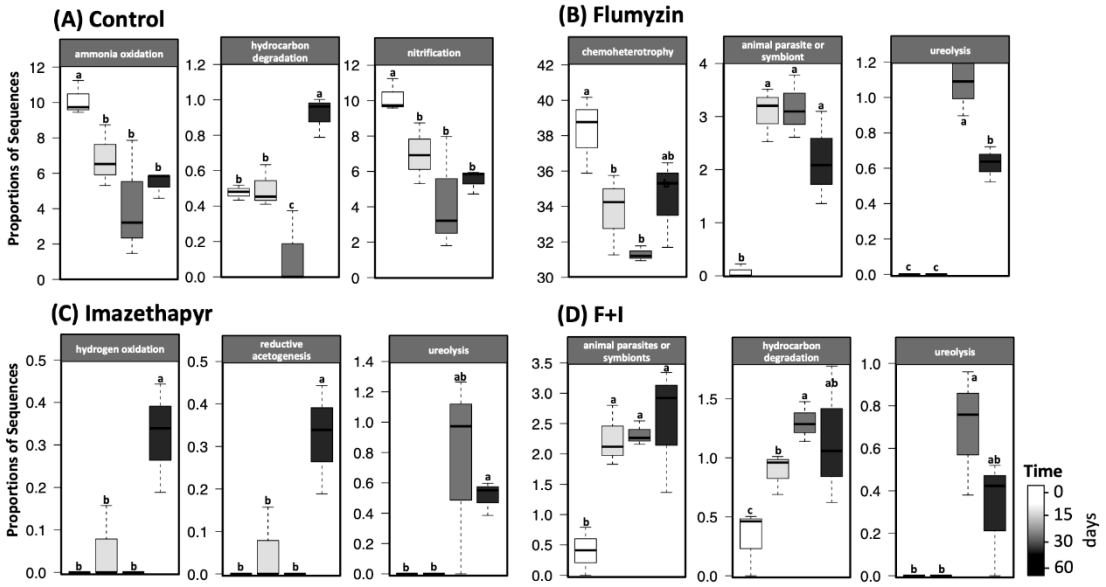

Figure 9. Effect of herbicide application on the abundance of potential bacterial functions during 60 days. The functions were predicted based on the bacterial and archaeal 16S rRNA gene using the FAPROTAX software. Only potential functions with a statistical difference by post-hoc Tukey-Kramer test after Benjamini-Hochberg correction ($P < 0.05$) are shown and indicated by different lower-case letters.

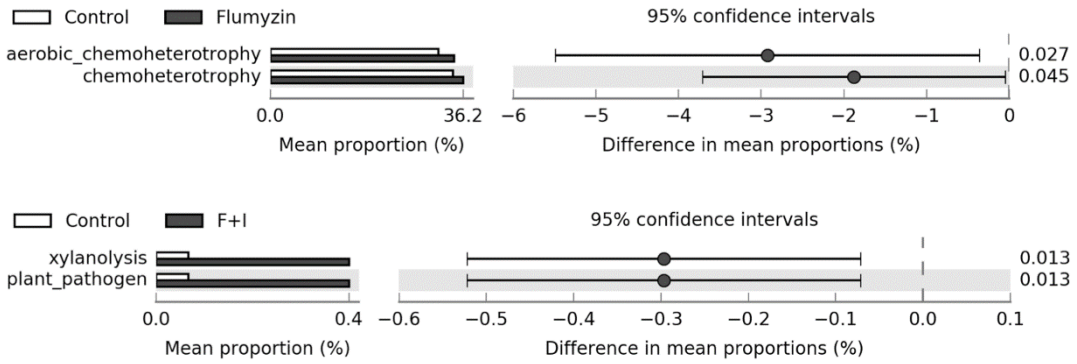

Figure 10. Scatter-plots comparing the abundance of predict functions between treatments. The functions were predicted based on the bacterial and archaeal 16S rRNA gene using the FAPROTAX software. Only potential functions with a statistical difference by post-hoc Tukey-Kramer test after Benjamini-Hochberg correction ($P<0.05$) are shown.

4.4. Discussion

4.4.1 Bacterial community structure, diversity, and composition

In this study, we used high-throughput sequencing of the 16S gene to assess the responses of bacterial community to the application of the herbicides imazethapyr and flumyzin, and their mixture, over 60 days after application. In a general view, the application of the herbicides increased the abundance of Gemmatimonadetes, Bacteroidetes, Firmicutes, and Proteobacteria. These results could indicate the ability of these groups in metabolizing the herbicides, increasing their abundance. Previous studies have shown Gemmatimonadetes, Bacteroidetes (Federici et al., 2017), Proteobacteria, and Firmicutes (Mijangos et al., 2009) as the main groups in soil treated with glyphosate.

Partially in line with our hypothesis, different herbicides did not affect differently the diversity but have a distinct influence on the composition of the microbial community in soil. The application of flumyzin increased the abundance of the genus *Bryobacter* and members of Acidimicrobiia and Acidobacteriales. The genus *Bryobacter* presents chemoorganotrophic activity and can use sugars, polysaccharides, and organic acids as an energy source (Dedysh et al., 2006), while members of Acidimicrobiales have the capability to assimilate energy from dimethylsulfoniopropionate, sulfonate, and carbon monoxide (Mizuno; Rodriguez-Valera; Ghai, 2015). Therefore, this result suggests that these microorganisms could be potential degraders of flumyzin.

On the other hand, the application of imazethapyr increased the abundance of

the genus *Gaiella,* which is strictly aerobic and chemoorganotrophic. A previous study has found *Gaiella* as the main degrader of the sulfadiazine (Chen et al., 2019). This genus is catalase- and oxidase-positive, presenting the capability for using several organic compounds as an energy source. Interestingly, *Gaiella* is catalase-positive and it could contribute to the degradation of imazethapyr since this herbicide belongs to the imidazolinones group that is degraded by catalase. Indeed, Perucci and Scarponi (1994) found imazethapyr increasing the activity of catalase in soil. Finally, when both herbicides were applied together, the genus *Conexibacterium* was positively influenced, increasing in abundance. This genus is known for its capability for surviving under stress conditions (Mann; Chen, 2010), being able to metabolize herbicides, such as triazine (Seki et al., 2012).

Interestingly, the genus *Gaiella* and a genus from the family Fimbriimonadaceae increased in abundance in all treatments with the application of the herbicides. As reported above, *Gaiella* presents positive activity of catalase that could contribute to degrading herbicides. Similarly, bacteria belonging to Fimbriimonas also present catalase-positive activity and these bacteria are found in contaminated soils (Im et al., 2012). According to Lü et al., (2009), catalase seems to be effective in degrading herbicides, presenting a potential strategy for biodegradation. Thus, the selection of bacterial groups with the capability for producing catalase could be important for herbicides degradation. For example, the genus *Flavobacterium* increased its abundance after the application of Flumyzin and Imazethapyr in mixture compared to the control, and this genus has been previously described as herbicide degrader, such as glyphosate (Chaudhry; Huang, 1988). An increased abundance of microbes capable to degrade herbicides would decrease the persistence and contamination of the soil by these chemical compounds. A lower persistence and rapid degradation of the herbicides in soils is required, once weeds are becoming more resistant to herbicides, which lead to an increase in its use (Singh; Singh, 2016).

The principal component analysis did not show a clear dissimilarity between treatments but indicated that the time influenced the responses of microbial communities in soil. Indeed, the diversity of bacteria and archaea varied according to the time of incubation, increasing during the first 15 days and presenting a decrease after this period. These results indicate a shift in the bacterial and archaeal communities according to the time of incubation and it could be related to the degradation time (DT), i.e. the persistence of these herbicides in soil. According to the

Pesticides Properties Data Base (2019), the DT of imazethapyr and flumyzin in similar soil types range from 7-19 and 15-20 days, respectively. Therefore, during the first 15 days, the bacterial and archaeal communities changed and increased their diversity to adapt and degrade the herbicides. After that period, the diversity decreased due to the dissipation of herbicides in the soil, which could explain the diversity variation during the incubation time. Similar results were reported by Moretto et al., (2017) who evaluated the effect of atrazine, diuron, and 2,4-D on bacterial community and found an influence of the persistence of each herbicide on the diversity of the bacterial community.

4.4.2 Functional profile of the bacterial community

We further analyzed the effect of the herbicides' application on the potential functional profile of the soil microbial communities. For this, the 16S rRNA data was affiliated to the FAPROTAX database for the functional prediction. The knowledge about the functional prediction is useful for understanding the relationship between microbial composition and their predicted functions, and these functional predictions have been used recently in several studies (Louca; Parfrev; Doebeli, 2016; Hariharan, 2017; Dube et al., 2019; Merloti et al., 2019; Rocha et al., 2020). Also, the study of bacterial functions in soils treated with chemical compounds can improve our understanding of the effect of these chemicals on the community dynamics, providing a basis for the compounds' degradation and restoration of contaminated ecosystems (Rocha et al., 2020). In our study, the dominant functional group was the chemoheterotrophs, indicating that the most proportion of microbes in our samples cannot fix carbon and have to obtain their energy through the oxidation of carbon molecules, such as carbohydrates and lipids (Zhang et al., 2018). Our analysis showed that the herbicides influenced differently the predicted functions and it can corroborate their effects on microbial composition. Compared to the control treatment, the application of the herbicide increased the abundance of chemoheterotroph microorganisms, indicating that a community that is able to degrade the herbicide is increasing in response to the presence of the chemical compound. The degradation of the herbicide can be accelerated due to the adaptation of the microbial population to that particular compound. However, the herbicide biodegradation in the soil also depends on the environmental conditions, such as temperature and moisture, which must be favorable for the development of a microbial community responsible for

degradation (Bundt et al., 2015). Thus, our analysis showed that the microbial community is responding to the herbicide application by increasing the abundance of chemoheterotrophs. Interestingly, the treatment with the application of flumioxazin presented a decrease of chemoheterotrophs along the time, probably as a response to the dissipation of the herbicide in the soil.

Also, the application of the herbicides in mixture increases the abundance of microbial groups with the potential function of xylanolysis. The xylanase is an enzyme that degrades the linear polysaccharide xylan into xylose, thus breaking down hemicellulose, a major component of plant cell walls (Beg et al., 2002). The increase of microbial groups linked to this function could be related to the increased availability of dead plant material after the application of the herbicides since xylanolysis plays a major role in microbes thriving on plant sources for the degradation of plant matter into usable nutrients. Indeed, the increase of xylanase activity was previously reported after the application of herbicide in soils with wheat (Wybieralshi; wybieralshi, 1988). In the treatment with the application of imazethapyr, there was an increase of microbial groups related to hydrogen oxidation and reductive acetogenesis, and both functions are dependent on the available hydrogen. The imazethapyr belongs to the imidazoline family that is excellent hydrogen donors (Xi et al., 2008), which explain the increase of microbial groups related to these functions after the herbicide application. Besides, the application of both herbicides in separated increased the abundance of microbial groups with potential function related to ureolysis. The ureolysis is related to ureolytic bacteria that use N and other nutrients to promote urease activity, being linked to biological activity (Ma et al., 2020). Previous studies have shown that these bacteria were stimulated with the application, in field rates, of triazine and pyrazosulfuron (Santric et al., 2014; Baboo et al., 2013). Since these herbicides contain N in their molecules, it may have contributed to increasing ureolysis in soil.

4.5. Conclusion

In summary, our study provides new information on the response of the soil bacterial community to the application of two herbicides separated or in mixture in an incubation period of 60 days. Our results revealed that the bacterial community was influenced by the time of application, presenting an increased diversity in the first 15 days and decreasing with time, which can be related to the degradation time of the herbicides. This finding suggests that the application of these herbicides, separated or in mixture, impacts the bacterial community during the two first weeks. Our results also showed an increase of microbial groups that have the potential to metabolize the chemical compounds, such as the genera *Bryobacter* and *Gaiella,* which increased significantly in abundance and can be explored for future biotechnological use. In addition, the prediction of the functional profile revealed an increase abundance of microbial groups with the potential to degrade the herbicides, such as chemoheterotrophs and xylanosis activity. Together, our study shows that the application of the herbicides in soil affects the microbial profile with the potential to affect functions mediated by microbial communities, and this effect can be related to groups with the potential to degrade the compounds. Specifically, the results from this study could help setting up an experiment to look more precisely at the response of *Bryobacter* and *Gaiella* in order to see if their metabolisms increase with the herbicide application.

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Supplementary Figures

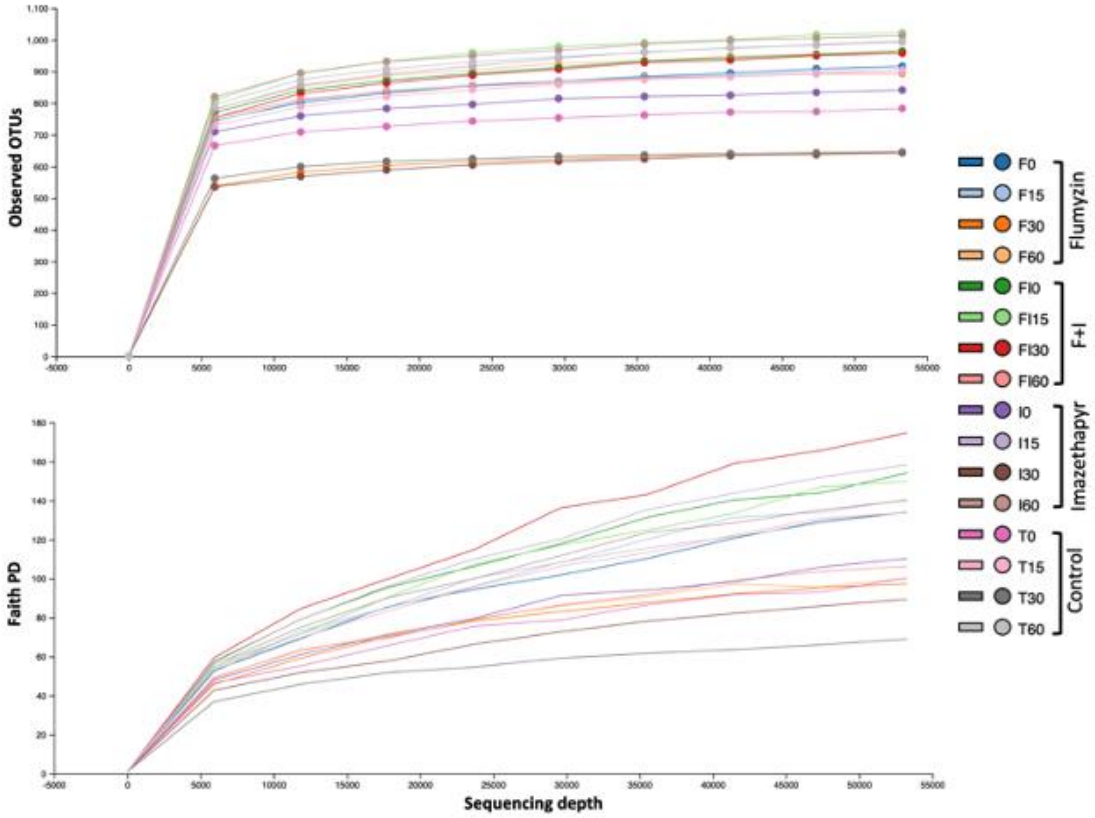

Figure S1. Rarefaction curves for the samples showing the sequencing depth based on the observed OTUs and Faith Phylogenetic Diversity.

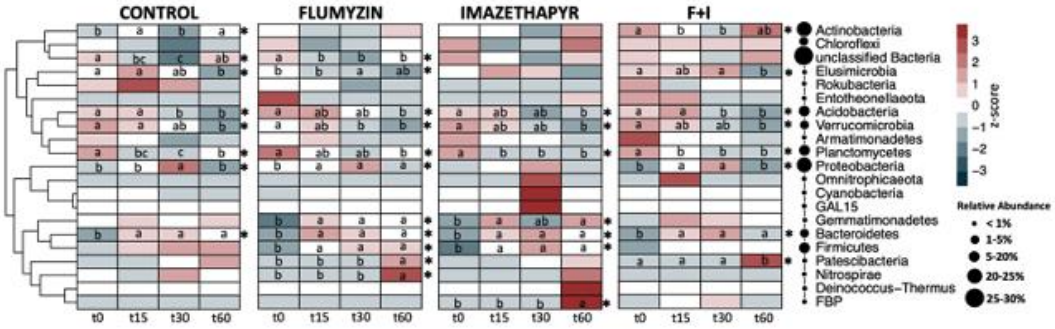

Figure S2. Heatmaps showing the differential abundance of bacterial phyla in soils with the application of herbicides along 60 days. The color key relates the heatmap colors to the standard score (z-score), i.e. the deviation from row mean in units of standard deviations above or below the mean. Asterisks and different lower case letters refer to significant differences between the days after the herbicide application within each treatment, based on Welch's t-test with Benjamini-Hochberg correction ($P < 0.05$). The relative abundance of microbial phyla is indicated by the size of each of the black circles.