

UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA VEGETAL

Thamara Ferreira Silva Ávila

**INIBIDORES DE UREASE DERIVADOS DE PRODUTOS NATURAIS E SEUS
EFEITOS SOBRE A MICROBIOTA DO SOLO, DESENVOLVIMENTO DE
Cenchrus americanus E CRESCIMENTO DE BACTÉRIAS PATOGÊNICAS**

BELO HORIZONTE – MG

2023

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Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Biologia Vegetal.

Orientadora: Prof^a. Dra. Luzia Valentina Modolo

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BELO HORIZONTE – MG

2023

- 043 Ávila, Thamara Ferreira Silva.
Inibidores de urease derivados de produtos naturais e seus efeitos sobre a microbiota do solo, desenvolvimento de *Cenchrus americanus* e crescimento de bactérias patogênicas [manuscrito] / Thamara Ferreira Silva Ávila. – 2023.
137 f. : il. ; 29,5 cm.
- Orientadora: Profa. Dra. Luzia Valentina Modolo. Coorientadores: Dr. Ângelo de Fátima; Dr. Ivanildo Evódio Marriel.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Biologia Vegetal.
1. Desenvolvimento Vegetal. 2. *Helicobacter pylori*. 3. Urease /antagonistas & inibidores. 4. Milhetes. I. Modolo, Luzia Valentina. II. Fátima, Ângelo de. III. Marriel, Ivanildo Evódio. IV. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. V. Título.
- CDU: 581



UNIVERSIDADE FEDERAL DE MINAS GERAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA VEGETAL
ICB-UFMG

FOLHA DE APROVAÇÃO

DEFESA DE TESE

THAMARA FERREIRA SILVA ÁVILA

DATA: 23/02/2023

TÍTULO DA TESE

"INIBIDORES DE UREASE DERIVADOS DE PRODUTOS NATURAIS E SEUS EFEITOS SOBRE A MICROBIOTA DO SOLO, DESENVOLVIMENTO DE CENCHRUS AMERICANUS E CRESCIMENTO DE BACTÉRIAS PATOGÊNICAS"

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Assinaturas dos membros da comissão examinadora

Assinatura do coordenador



Documento assinado eletronicamente por André Marques dos Santos, Usuário Externo, em 23/02/2023, às 18:56, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por Luzia Valentina Modolo, Professora do Magistério Superior, em 27/02/2023, às 07:30, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por Simone Raposo Cotta, Usuária Externa, em 27/02/2023, às 10:11, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por Reinaldo Bertola Cantarutti, Usuário Externo, em 27/02/2023, às 12:01, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por Krisle da Silva, Usuária Externa, em 27/02/2023, às 17:05, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



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Aos meus pais, Nailda e Sebastião,
Dedico.

Agradecimentos

Agradeço primeiramente a Deus, pela bênção da vida. Agradeço aos meus pais, à minha mãe, Nailda Conceição e ao meu pai, Sebastião Francisco, pela vida, pelo exemplo e por toda luta, incentivo e apoio. Agradeço à minha família, pelos inúmeros momentos de alegria, convivência e aprendizado. Em especial, aos meus irmãos, Thales, Nathaly e Nicolás, por todas as conversas agradáveis e convívio. Nathaly e meu cunhado Douglas, por toda a imensurável ajuda. À minha avó, carinhosamente chamada de mãe preta, por todo cuidado comigo e com minha filha, Helena. Nilma, Sofia, Edgar, Edemilson, Onofre Filho, Neire, Livia, muito obrigada a todos. Agradeço, especialmente, meu esposo Geraldo, por todo incentivo, luta e paciência. À minha cunhada Heloísa, obrigada por todo o apoio. À minha filha Helena, obrigada por iluminar meu caminho.

Um agradecimento especial à minha orientadora e aos meus coorientadores: Luzia Valentina Modolo, Ângelo de Fátima e Ivanildo Evódio Marriel. Muito obrigada por todas as oportunidades de aprendizado, pela paciência, pelas reuniões e conversas. Agradeço aos demais membros da REDNIU e GEBioPlan, atuais e ex, Carol Dohanik, Breno, Débora, Sarah, Ana Cláudia, Samuel, Clara, Lara, Andreia, Luisa foi um prazer conviver com cada um de vocês.

Agradeço também a todos os professores que participaram do trabalho, seja por meio de parcerias ou outras contribuições. Ao professor Rodrigo Kitagawa, da Universidade Federal do Espírito Santo, ao professor Ricardo José Alves da UFMG e ao Flaviano Ottoni, pela parceria e às contribuições com os estudos relacionados aos derivados naftoquinônicos. Ao professor Ari Goes Neto da Universidade Federal de Minas Gerais e ao Glen Garcia pela contribuição com estudo metagenômico. À Yumi, Bárbara e Renata do Departamento de Ecologia, pela concessão do espaço da casa de vegetação. Agradeço ao técnico, do Departamento de Bioquímica, Jamil Oliveira, muito obrigada por toda a ajuda. Aos amigos Tiago Vilas Boas e Aldineia Buss, obrigada pelas conversas e contribuições. Agradeço à UFMG e ao Programa de pós-graduação em biologia vegetal. À CAPES pela concessão da bolsa, ao CNPq e à FAPEMIG pelo apoio financeiro à pesquisa.

Resumo geral

Inibidores de urease constituem uma opção para atenuar a volatilização da amônia no uso da ureia como fertilizante com consequente aumento da produtividade agrícola e a mitigação dos impactos ambientais. Inibidores de urease têm também emergido como alternativa para o tratamento de infecção por *Helicobacter pylori*, já que a urease é um dos fatores de virulência desta bactéria. O presente trabalho descreve os efeitos de novos inibidores de urease derivados de aminoácidos nas ureases do solo, na microbiota do solo e no metabolismo e desenvolvimento de milho. Além disso, é relatado o potencial de 30 derivados do produto natural lapachol e lausona para inibir *in vitro* a urease de *Canavalia ensiformis*, o crescimento de *H. pylori* e citotoxicidade contra células de tumores gástricos. Quatro de 13 derivados de aminoácidos avaliados apresentaram porcentagem de inibição na mesma faixa que o inibidor referência NBPT (49 - 75%). Dois derivados estimularam o acúmulo de biomassa em raízes de milho cultivados por 35 dias em casa de vegetação e o acúmulo de nutrientes minerais na parte aérea. O aumento da performance vegetal desencadeado pelos derivados de aminoácidos foi acompanhado de um aumento da fragilidade do microbioma, uma vez que ambos os compostos promoveram uma fragmentação da rede de interação microbiana na rizosfera de milho. Cinco dos 30 derivados do lapachol e da lausona inibiram completamente a atividade da urease de *Canavalia ensiformis*, inibiram o crescimento de *H. pylori* (64 - 256 µg/mL) e apresentaram IC₅₀ menores ou iguais a 68.8 µg/mL quanto ao crescimento de células de tumores gástricos. O aumento da performance vegetal desencadeado pelos derivados de aminoácidos foi acompanhado de uma fragmentação da rede de interação microbiana na rizosfera de milho. Contudo, ao final do experimento, a comunidade bacteriana mostrou-se resiliente ao tratamento, indicado que os derivados de aminoácidos não são deletérios à população bacteriana presente no solo. Derivados do lapachol e da lausona são moléculas promissoras para futuras investigações para tratamento de carcinoma gástrico e de infecção por *Helicobacter pylori*.

Palavras-chave: Desenvolvimento de milho, *Helicobacter pylori*, Inibidores de urease, metabolismo vegetal, metagenômica, microbiota edáfica, Ureia.

General abstract

Urease inhibitors constitute an option to mitigate ammonia volatilization in the use of urea as a fertilizer, resulting in increased agricultural productivity and the mitigation of environmental impacts. Likewise, urease inhibitors have emerged as an alternative for the treatment of infections caused by *Helicobacter pylori*, as the urease is one of the virulence factors for such a bacterium. This work reports the effect of new urease inhibitors derived from amino acids on soil ureases, soil microbiota and metabolism and growth of millet plants. It is also shown evidence of the potential of some lapachol and lawsone glucoderivatives to inhibit the *Canavalia ensiformis* urease *in vitro* and the growth of *H. pylori* and proliferation of gastric tumor cells. Four out of 13 amino acid derivatives were as efficient as NBPT towards soil ureases (inhibition from 49 to 75%). Two amino acid derivatives stimulated the accumulation of biomass in millet roots grown for 35 days in a greenhouse and the accumulation of mineral nutrients in millet shoot. The increment in millet performance caused by some amino acid derivatives was accompanied of an increase in the soil microbiome sensitivity, due to the stimulation of the fragmentation of the microbiota network interaction in the millet rhizosphere. Five out of 30 lapachol and/or lawsone glucoderivatives completely inhibited the activity of *Canavalia ensiformis* urease *in vitro*, the *H. pylori* growth (MIC in the range from 64 to 256 µg/mL) and IC₅₀ values equals or lower than 68.8 µg/mL toward gastric cancer cells. The increase in plant performance triggered by amino acid derivatives was accompanied by a fragmentation of the microbial interaction network in millet rhizosphere. However, at the end of the experiment, the bacterial community proved to be resilient to the treatment, indicating that amino acid derivatives are not deleterious to the bacterial population present in the soil. Derivatives of lapachol and lausone are promising molecules for future investigations for the treatment of gastric carcinoma and *Helicobacter pylori* infection.

Keywords: Millet development, *Helicobacter pylori*, Urease inhibitors, plant metabolism, metagenomics, edaphic microbiota, Urea.

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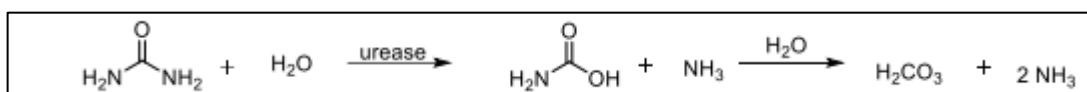
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Introdução Geral

Ureases são enzimas hidrolíticas relacionadas ao bem estar humano e com o meio ambiente (Mazzei et al., 2019). Esta enzima catalisa a conversão de ureia em amônia e carbamato, o qual é degradado a amônia e ácido carbônico (Krajewska, 2009) conforme sumarizado na reação descrita a seguir:



Ureases são produzidas por bactérias, fungos, plantas e alguns invertebrados. A urease foi a primeira enzima a ser cristalizada (Sumner, 1926), sendo importante na descoberta do papel do níquel em sistemas biológicos devido à sua dependência de Ni^{2+} para catálise da hidrólise de ureia (Dixon et al., 1975; Dixon et al., 1980). A descoberta da correlação entre a presença de *Helicobacter pylori* no trato gastroduodenal e a incidência de úlceras gástricas resultou na concessão do Prêmio Nobel a Barry Marshall e Robin Warren em 2005. Marshall desenvolveu ainda o primeiro teste clínico baseado na atividade da urease capaz de identificar a presença de *Helicobacter pylori*.

Ureases constituem um dos fatores de virulência associados à sobrevivência de patógenos como *Helicobacter pylori* em meios muito ácido, como os do estômago. Isso ocorre, porque a amônia liberada a partir da hidrólise da ureia presente no suco gástrico, captura prótons, permitindo a formação de um microambiente menos ácido, em torno do sítio de infecção (Shibata et al., 2006) propício ao crescimento da bactéria patogênica. Estima-se que mais da metade da população mundial esteja infectada com *H. pylori* (Mladenova, 2021). Além disso, a infecção por *H. pylori* é um importante fator patogênico para gastrite crônica, úlcera e câncer gástrico (Blaser et al., 1995; Muhammad et al., 2019). Dessa forma, muitas pesquisas têm feito esforços na busca de composto inibidores dessa enzima, como alvo adicional, para o tratamento dessa infecção (Kataria and Khatkar, 2019).

Os microrganismos do solo produzem ureases e as secretam para a solução do solo. Ureases extracelulares são adsorvidas pela matriz do solo, o que garante maior persistência dessa enzima e correspondem a 79 - 89 % da atividade de ureases no solo (Paulson & Kurtz, 1969). A elevada atividade de ureases no solo proporciona perda da eficiência do adubo ureia, uma vez que quando lançada ao solo, por

cobertura, é rapidamente hidrolisada (Upadhyay, 2012). Em casos extremos, a perda de ureia por volatilização de amônia pode chegar a 80 % (Santos et al., 2016 Cantarella et al; 2018). Em solos brasileiros é comum perdas por volatilização em torno de 30 % da ureia aplicada (Cantarella et al., 2008).

A ureia é o principal adubo nitrogenado utilizado mundialmente (IFASTAT, 2022) devido ao elevado teor de N (46%), facilidade de aplicação e menor custo relativo de produção (Soares et al., 2012; Saggar et al., 2013). O uso de inibidores de urease em formulações à base de ureia tem sido usado como estratégia para diminuir a volatilização de amônia e consequentemente reduzir as perdas de N. Tal prática possibilita a melhor incorporação da ureia ao solo, permitindo maior oferta de N ao sistema radicular da planta (Dawar et al., 2021).

A fim de reduzir as perdas de N, a União Europeia exige que a ureia aplicada ou incorporada no solo seja combinada com inibidor(es) de urease (União Europeia, 2019). Cerca de 30 % da ureia aplicada mundialmente, utiliza alguma tecnologia para aumentar sua eficiência, sendo que somente cerca de 16 % da ureia aplicada é protegida com inibidor de urease (IFASTAT, 2019). Ou seja, há uma tendência e necessidade no aumento do uso de fertilizantes com eficiência aumentada.

NBPT (triamida *N*-butiltiofosfórica) é o inibidor de urease mais utilizado mundialmente, comercializado desde do início dos anos de 1990 nos Estados Unidos. O NBPT é um análogo da ureia e sua estabilidade à temperatura e no solo, principalmente naqueles relativamente ácidos, como os solos brasileiros, é bastante comprometida (Lasisi and Akinremi, 2022). No entanto, os principais produtos oriundos da metabolização do NBPT no solo também contribuem para inibição das ureases do solo (Peters and Thiele-Bruhn, 2022). No Brasil, a compra desse insumo importado onera os custos da produção agrícola.

O Brasil é um dos principais fornecedores mundiais de produtos agrícolas, sendo que cerca de 28% do seu território é atualmente utilizado com atividades agropecuárias (MAPA, 2019). O Brasil possui também a maior biodiversidade mundial, além de abrigar a maior floresta tropical do mundo (Valli et al., 2019). Dessa forma, para que ocorra o aumento da produtividade sem expansão da fronteira agrícola, o desenvolvimento tecnológico torna-se uma ótima estratégia. Estudos demonstram que o aumento da produtividade agrícola brasileira, é devido em 68 % pelo emprego de tecnologia, seguido de 23 % devido às relações de trabalho e somente em 9 % pela expansão da fronteira agrícola (Souza e Rocha, 2012). A maior consciência

global, nos tempos atuais, da importância da preservação ambiental claramente indica que a expansão de terras agricultáveis não é uma opção para aumentar a produção agrícola. Dessa forma, é crucial o desenvolvimento tecnológico nacional, voltado para o setor agrícola, principalmente no que tange fertilizantes de eficiência aumentada. É importante, porém, que as novas substâncias promissoras para aplicações tecnológicas sejam compostos que apresentem ação inibitória de ureases e que não sejam prejudiciais às culturas vegetais e ao meio ambiente (Modolo et al., 2018). A saúde do solo é extremamente importante para manutenção dos serviços ecossistêmicos e para o bom desempenho das culturas (Bargali et al., 2019; Wei et al., 2019). Além disso, vem sendo demonstrado cada vez mais, a relação positiva entre atividade microbiana e aumento da produtividade (Dotaniya et al., 2019).

Novas substâncias têm sido descritas como inibidores de urease e exemplos incluem compostos das seguintes classes: fosforamidatos (Oliveira et al., 2014), benzotiazóis (Araújo et al., 2015) e benzoiltioureas (Brito et al., 2015). Substâncias derivadas de aminoácidos também foram descritas como potenciais candidatas a inibidores de urease (Otake et al., 1992). Dezoito substâncias derivadas da glicina mostraram-se como potentes inibidores da urease de *Canavalia ensiformis* (IC₅₀ na faixa de 1,8 a 20 µM). Mais recentemente, Ullah et al. (2018) sintetizaram 21 compostos híbridos contendo D-valina ou D-triptofano, que também apresentaram expressiva atividade anti-ureolítica contra a urease de *Bacillus pasteurii*.

Apesar do número relativamente expressivo de substâncias do potencial para inibir ureases, estudos visando investigar o efeito dessas substâncias em vegetais e microbiota do solo são escassos. Dessa forma, esse trabalho buscou avaliar o potencial de derivados de aminoácidos e derivados dos produtos naturais lapachol e lausona para inibir ureases. (Tio)hidantoínas, derivadas de aminoácidos, foram avaliadas quanto a capacidade de inibir ureases do solo, assim como, seus efeitos na microbiota do solo e desenvolvimento e metabolismo vegetal. Naftoquinonas derivadas do lapachol e da lausona, foram avaliadas quanto à capacidade de inibição de ureases purificadas e de ureases do solo, além de triagem inicial para possíveis aplicações no tratamento da infecção por *H. pylori* e de tumores gástricos.

Objetivos

Objetivo geral

O objetivo geral do trabalho foi identificar novos inibidores de urease derivados de produtos naturais de interesse agrícola e medicinal e avaliar a toxicidade dos mesmos sobre plantas e microbiota do solo.

Objetivos específicos

Capítulo 1

Avaliar a capacidade de derivados de aminoácidos em inibir ureases do solo, determinar seus efeitos sobre indicadores de saúde do solo e sobre o desenvolvimento de milho cultivado sob condições controladas;

Capítulo 2

Avaliar o efeito dos inibidores de urease de derivados de aminoácidos mais eficientes sobre a germinação de sementes, o desenvolvimento inicial e o metabolismo antioxidante de milho;

Capítulo 3

Determinar o efeito dos inibidores D-106 e D-Ac-106 a baixas concentrações no desenvolvimento inicial de milho cultivado na presença de diferentes fontes de nitrogênio. Avaliar *in vitro* o efeito dos exsudatos radiculares oriundos das plantas tratadas no potencial de nitrificação do solo;

Capítulo 4

Avaliar o efeito dos inibidores D-106 e D-Ac-106 na performance de milho cultivado em vaso e na diversidade e estrutura do microbioma rizosférico de milho;

Capítulo 5

Avaliar a capacidade de 30 derivados das naftoquinonas lapachol e lausona para inibir *in vitro* a urease de *Canavalia ensiformis*, bem como o crescimento *in vitro* de uma cepa de *Helicobacter pylori* resistente a antibiótico e de células de tumores gástricos.

Referências

- Alves, E.; Souza, G. S.; Rocha, D. P. Lucratividade na agricultura. (2012). *Revista de Política Agrícola*, v. 21, n. 2, p. 45-63.
- Araújo, D. P., Morais, S, V. S. S., de Fátima, A., Modolo, L. V. (2015). Efficient sodium bisulfite-catalyzed synthesis of benzothiazoles and their potential as ureases inhibitors. *RSC Advances: an international journal to further the chemical sciences*, v. 5, p. 28814-28821.
- Bargali, S. S., Padalia, K., & Bargali, K. (2019). Effects of tree fostering on soil health and microbial biomass under different land use systems in the Central Himalayas. *Land Degradation & Development*, 30(16), 1984-1998.
- Blaser, M. J., Perez-Perez, G. I., Kleanthous, H., Cover, T. L., Peek, R. M., Chyou, P. H., ... & Nomura, A. (1995). Infection with *Helicobacter pylori* strains possessing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer research*, 55(10), 2111-2115.
- Cantarella, H., Trivelin, P. C. O., Contin, T. L. M., Dias, F. L. F., Rossetto, R., Marcelino, R., ... & Quaggio, J. A. (2008). Ammonia volatilisation from urease inhibitor-treated urea applied to sugarcane trash blankets. *Scientia agrícola*, 65, 397-401.
- Cantarella, H., Otto, R., Soares, J. R., & de Brito Silva, A. G. (2018). Agronomic efficiency of NBPT as a urease inhibitor: A review. *Journal of advanced research*, 13, 19-27.
- Dawar, K., Fahad, S., Jahangir, M. M. R., Munir, I., Alam, S. S., Khan, S. A., ... & Danish, S. (2021). Biochar and urease inhibitor mitigate NH₃ and N₂O emissions and improve wheat yield in a urea fertilized alkaline soil. *Scientific reports*, 11(1), 1-11.
- Dixon, N. E.; Gazzola, C.; Blakeley, R. L.; Zerner, B. (1975). Jack bean urease (EC3.5.1.5). Metalloenzyme. Simple biological role for nickel. *Journal of the American Chemical Society*, Easton, v. 97, n. 14, p. 4131-4133, 1975.
- Dixon, N. E.; Hinds, J. A.; Fihelly, A. K.; Gazzola, C. et al. (1980). Jack bean urease (EC 3.5.1.5). IV. The molecular size and the mechanism of inhibition by hydroxamic acids. Spectrophotometric titration of enzymes with reversible inhibitors. *Can J Biochem*, [s. l.], v. 58, n. 12, p. 1323-1334, Dec 1980.
- Dotaniya, M. L., Aparna, K., Dotaniya, C. K., Singh, M., & Regar, K. L. (2019). Role of soil enzymes in sustainable crop production. In *Enzymes in food biotechnology*

- (pp. 569-589). Academic Press.
- IFASTAT and Association, (2019). Association, I.F. (Ed.), 1. Urease Inhibitors - Consumption, Paris, France. <https://www.ifastat.org/databases/graph/1>
- IFASTAT and Association, (2022). Association, I.F. (Ed.), 1.1 Fertilizer Consumption - Historical Trends by Country or Region, Paris, France, https://www.ifastat.org/databases/graph/1_1
- Kataria, R., & Khatkar, A. (2019). Molecular docking, synthesis, kinetics study, structure–activity relationship and ADMET analysis of morin analogous as *Helicobacter pylori* urease inhibitors. *BMC chemistry*, 13(1), 1-17.
- Lasisi, A. A., & Akinremi, O. O. (2022). Degradation of N-(n-butyl) Thiophosphoric Triamide (NBPT) with and without Nitrification Inhibitor in Soils. *Nitrogen*, 3(2), 161-169.
- MAPA- Ministério da Agricultura, Pecuária e Abastecimento. Agropecuária brasileira em números - Abril de 2019. Disponível em: <<http://www.agricultura.gov.br/assuntos/politica-agricola/todas-publicacoes-de-politica-agricola/agropecuaria-brasileira-em-numeros/ABN201904/view>>. Acesso em: 15. Mai. 2019.
- Mazzei, L., Cianci, M., Benini, S., & Ciurli, S. (2019). The structure of the elusive urease–urea complex unveils the mechanism of a paradigmatic nickel-dependent enzyme. *Angewandte Chemie International Edition*, 58(22), 7415-7419.
- Modolo, L.V, Da-Silva, C.J., Brandão, D.S., Chaves, I.S. (2018). A minireview on what we have learned about urease inhibitors of agricultural interest since mid-2000s. *Journal of Advanced Research*. 13, 29-37.
- Muhammad, J. S., Eladl, M. A., & Khoder, G. (2019). *Helicobacter pylori*-induced DNA methylation as an epigenetic modulator of gastric cancer: recent outcomes and future direction. *Pathogens*, 8(1), 23.
- Mladenova, I. (2021). Clinical relevance of *Helicobacter pylori* infection. *Journal of Clinical Medicine*, 10(16), 3473.
- Odake, S., Nakahashi, K., Morikawa, T., Takebe, S., & Kobashi, K. (1992). Inhibition of urease activity by dipeptidyl hydroxamic acids. *Chemical and pharmaceutical bulletin*, 40(10), 2764-2768.
- Oliveira, F. M., Barbosa, L. C. A., Demuner, A. J., Maltha, C. R. A., Pereira, S. R., Horta, L. P., Modolo, L. V. (2014). Synthesis, molecular properties and DFT studies of new phosphoramidates as potential urease inhibitors. *Medicinal*

- Chemistry Research. 23, 5174-5187.
- Paulson, K. N., & Kurtz, L. T. (1969). Locus of Urease Activity in Soil¹. Soil Science Society of America Journal, 33(6), 897.
- Peters, N., & Thiele-Bruhn, S. (2022). Major metabolites of NBPT degradation pathways contribute to urease inhibition in soil. Chemosphere, 135163.
- Santos, S.M.C. dos, Antonangelo, J.A, Deus, A.C.F., Fernandes, D.M. (2016). Perdas de amônia por volatilização em resposta a adubação nitrogenada do feijoeiro. Revista de Agricultura Neotropical. 3(1): 16-20.
- Ruser, R., & Schulz, R. (2015). The effect of nitrification inhibitors on the nitrous oxide (N₂O) release from agricultural soils—a review. Journal of Plant Nutrition and Soil Science, 178(2), 171-188.
- Saggar, S. Singh, J. Giltrap, D.L, Zaman, M. Luo, J. Rollo, M. Kim, D.G. Rys, G. Van der Weerden T.J. (2013) Quantification of reductions in ammonia emissions from fertiliser urea and animal urine in grazed pastures with urease inhibitors for agriculture inventory: New Zealand as a case study. Sci. Total Environ. 465:136-146.
- Shibata T, Imoto I, Taguchi Y, Takaji S, Ikemura N, Nakao K, et al. (1996). High acid secretion may protect the gastric mucosa from injury caused by ammonia produced by *Helicobacter pylori* in duodenal ulcer patients. J Gastroenterol Hepatol 11:674–80.
- Upadhyay, L.S. (2012). Urease inhibitors: A review. Indian Journal of Biotechnology. 11, 381–388.
- European Union, (2019). Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 Laying Down Rules on the Making Available on the Market of EU Fertilising Products and Amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and Repealing Regulation (EC) No 2003/2003. European Parliament and the Council of the European Union, Brussels.
- Sumner, J. B. (1926). Note. The recrystallization of urease. Journal of Biological Chemistry, 70(1), 97-98.
- Valli, M., & Bolzani, V. S. (2019). Natural products: perspectives and challenges for use of Brazilian plant species in the bioeconomy. Anais da Academia Brasileira de Ciências, 91.
- Wei, Z., Gu, Y., Friman, V. P., Kowalchuk, G. A., Xu, Y., Shen, Q., & Jousset, A. (2019). Initial soil microbiome composition and functioning predetermine future plant

health. *Science advances*, 5(9), eaaw0759.

Chapter 1

Do amino acid-derived urease inhibitors affect the activity of the soil microbiota and millet (*Cenchrus americanus*) growth?

Abstract

Nitrogen fertilization is extremely important for increasing agricultural productivity. However, crops absorb less than 50 % of the applied nitrogen, since much of it is lost through processes such as volatilization, denitrification and leaching. The use of urease inhibitors is one of the strategies adopted to reduce these losses and to minimize the environmental impacts resulting from this fertilization. Thus, this study evaluated the impact of urea application and new inhibitors derived from amino acids and the commercial inhibitor *N*-(butyl) thiophosphoric triamide (NBPT) on microbial properties related to soil quality indicators and on *Cenchrus americanus* growing in a greenhouse. For this, properties such as soil basal respiration, microbial biomass, metabolic quotient and fluorescein diacetate hydrolysis in soil (Latossolo vermelho distrófico) with or without rhizospheric influence were evaluated. The application of urea and urease inhibitors had no significant effects at the end of the analysis on microbiological properties. The inhibitors also showed no apparent negative effect on plant growth. The D-Ac-106 compound stimulated a greater contribution of biomass to the root system and stimulated greater growth of the primary root in *Cenchrus americanus* seedlings. Thus, these substances have great potential to be commercially exploited as urease inhibitors, since they have satisfactory inhibitory action, in addition to bringing advantages promoting better development of the root system, which can provide better exploitation of the soil by resources such as water and nutrients.

Keywords: *Cenchrus americanus* development, Urease inhibitors, Soil microbiota, Soil quality.

1. Introduction

One of the main issues of the 21st century is food production, especially when considering the current scenario of climate change and the growing world population. The high productivity in the last 50 years has been guaranteed by the large amounts of fertilizers applied to the soil. However, soil fertilization brought many problems such as environmental pollution and soil deterioration (Beretta-Blanco et al., 2019). The development of new technologies has allowed better efficiency in the use of nutrients (Abdalla et al., 2022). Urease inhibitors comprise one technology that delays urea hydrolysis into ammonia and carbon dioxide allowing urea to be incorporated into the soil and thus avoiding N losses. On average, 50% of the urea worldwide applied in the soil is lost due to ammonia volatilization. Currently, the urease inhibitor NBPT (*N*-butyl thiophosphoric triamide), a structural analogue of urea, is the most worldwide used in agriculture to overcome N losses from urea. It shows substantial degradation at temperatures greater than 25°C (Watson et al., 2008), and in acidic and slightly alkaline soils (Engel et al., 2015). In alkaline soils, NBPT also stimulates greater release of nitrous oxide into the atmosphere, a gas that currently contributes to the worsening of the greenhouse effect (Fan et al., 2018). New and more environmentally friendly substances have been investigated for their potential as urease inhibitors (Modolo et al., 2018).

Examples of urease inhibitors include urea analogues (hydroxymates and derivatives), thiols (Trenkel, 2010), phosphoramidates (Oliveira et al., 2014), benzothiazoles (Araújo et al., 2015) and benzoylthioureas (Brito et al., 2015). Amino acid derivatives have also been described as potential candidates for urease inhibitors (Otake et al, 1992; Camargo et al., 2022). Eighteen glycine-derivatives were shown to be effective against *Canavalia ensiformis* urease (IC_{50} from 1.8 to 20 μ M). Twenty one D-valine or D-tryptophan derivatives also showed significant anti-ureolytic activity against the *Bacillus pasteurii* urease. Similarly, Camargo et al, (2022) demonstrated a high capacity of (thio)hydantoins derived from L-Valine to inhibit the *Canavalia ensiformis* urease. However, the effect of urease inhibitors based on amino acid derivatives on soil microbiota and plants remains to be disclosed. Ideally, a good new urease inhibitor should be efficient against ureases and safe to be used in soil besides not being harmful to plants and the environment (Modolo et al., 2018).

Thus, the biomonitoring of soil quality indicators is important to attest to the safety of a certain urease inhibitor. Microbial biomass, microbial respiration and soil enzymatic activity are among the bioindicators to be considered in this context. Such biochemical indicators are used to assess management systems and the impact of the introduction of xenobiotics in the soil (Gianfreda and Rao, 2017). This present study aimed to evaluate the effect of urease inhibitors derived from amino acids on soil quality properties and the growth of *Cenchrus americanus* (millet). The structure of the compounds is shown in Figure 1, except for the racemate (L/D) corresponding to the product obtained from the racemic mixture of the corresponding amino acid.

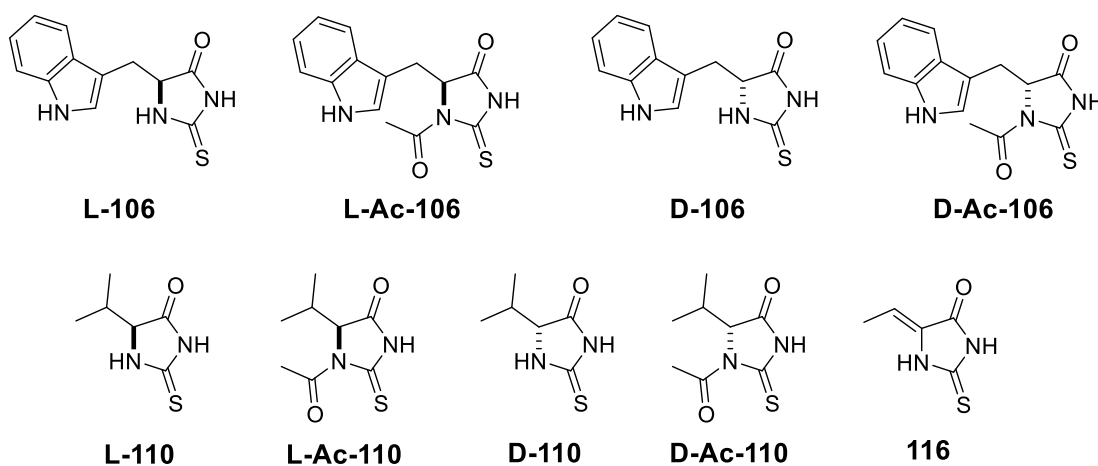


Figure 1. Structures of acetyl (thio)hydantoin and thiohydantoin.

2. Material and methods

2.1 Inhibition of soil ureases

The screening of compounds was carried out in samples of a Latossolo vermelho distrófico soil collected in the Sete Lagoas city, Minas Gerais, in an experimental area of Embrapa Milho and Sorghum. The soil (0.5 g) was incubated at 37 °C for 1 h with 72 mM urea in the presence or absence of each inhibitor at 500 µM. The NBPT was used as a reference of urease inhibitor. Reactions were stopped by adding a solution of 1 M KCl in 10 mM HCl, followed by incubation at 25 °C for 30 min. After centrifugation, a fraction of the supernatant was collected and added to a solution containing 3.4 % w/v sodium salicylate, 2.5 % w/v sodium citrate, 2.5 % w/v sodium tartrate and sodium nitroprusside. 1.2×10^{-4} % m/v. The mixtures were then incubated at 25 °C and 600 rpm in the dark for 15 min, followed by addition of 3 % w/v sodium hydroxide and 1 % v/v sodium hypochlorite. The systems were incubated at 25 °C and 600 rpm in the dark for 1 h and analyzed at 660 nm to quantify the NH_4^+ formed (Kandeler and Gerber, 1988).

2.2 Experimental design

Four out of 12 compounds initially tested on soil (D-Ac-106, D-106, L-106 and 116) were selected due to their efficiency to inhibit ureases for the greenhouse randomized tests. Firstly, an experiment with millet was carried out in vessels to analyze the effect of the compounds on the soil and on plant development and growth. The second experiment was carried out with soil in the presence of compound. A sieved (2 mm) latossolo vermelho distrófico soil collected from an experimental area of EMBRAPA Milho and Sorghum Sete Lagoas/MG (19°28'01.2"S and 44°10'24.5"W) was used in all experiments. The physical and chemical characteristics of the soil were 19.2 % sand, 10.3 % silt, 70.5% clay, pH(H₂O) 6.14, N 0.171 dag kg⁻¹, PMehlich 18.6 mg dm⁻³, KMehlich 215 mg dm⁻³, Ca²⁺ 7.85 cmol_c dm⁻³, Mg²⁺ 0.97 cmol_c dm⁻³, Al absent, H⁺ Al 2.5 cmol_c dm⁻³, organic matter 4.43 dag kg⁻¹ and 78.9 % base saturation. Thus, in experiment 1, sowing was carried out in 5-liter pots, with 4 kg of soil and 8 plants each. Thinning was carried out to keep only the plants with uniform growth in the pots. The soil moisture was maintained at 75 % of field capacity. The plants (15 days after sowing) received treatment with or without urea (150 mg de N/ Kg of soil) and urea plus inhibitors at concentrations of 0.06 % or 1 % (w/w) in relation to weight

of urea. The compounds were applied as aqueous solutions prepared in 0.015 % ethanol, 0.015 % DMSO and 0.5 % polyethylene glycol 400 to ensure homogeneous distribution in the soil. Rhizosphere soil samples were collected at 10 cm from the surface from the pots 24 h before treatments and 1 day, 3 days, 7 days, 15 days and 35 days post treatments. For this, the plants were removed and the soil close to the root collected in a sterile plastic bag and immediately stored in a refrigerated styrofoam box. The soil samples were then sieved in a 2 mm mesh sieve and stored at 4 °C until the time of analysis. At the end of the experiment, the height of the plants was measured, followed by the collection of the root system, stem and leaves, which were dried in an oven with forced air circulation at 65 °C to estimate the accumulated biomass. The second experiment (performed subsequent to the first) contained soil devoid of plants, received the same treatments and followed all the harvesting procedures as described for the first experiment.

2.3 Estimation of the content of chloroplast pigments

To determine the amounts of chloroplast pigments, 0.1 g of fresh material, collected at the end of the experiment, was immersed, ground in 5 mL of 80 % acetone and centrifuged at 4 °C. The levels of pigments present in the extracts were measured at 647 and 663 nm to calculate the amount of chlorophylls *a* and *b*, respectively. Measurements at wavelength 470 nm were used to calculate the levels of carotenoids, according to Lichtenthaler (1987).

2.4 Basal soil respiration

Basal respiration was determined by the quantification of CO₂ released from the soil microbiota, as described by Alef and Nannipieri (1995). Briefly, 40 g of soil were placed in airtight glass pots 1,5 L. A flask containing 10 mL of 0.5 M NaOH solution was placed inside the pot next to the soil. Samples were incubated in the dark at 28 °C for 72 h. Afterwards, 2 mL of 25% BaCl₂ (w/v) and three drops of 1% phenolphthalein were added to the flask containing 0.5 M NaOH. These samples were titrated with 0.5 M HCl. Basal soil respiration (BSR) was determined according to the equation: $BSR_{(\mu g\ C-CO_2\ h^{-1}\ g^{-1}\ soil)} = \frac{[(V_b - V_a) \times C_{HCl} \times 6 \times 1000] \div m_s}{T}$, where *V_b* corresponds to the volume of HCl (mL) spent in standardizing the NaOH concentration, *V_a* corresponds to the volume of HCl (mL) spent to titrate the sample, *C_{HCl}* is the precise

concentration of HCl (mol L^{-1}), m_s correspond to the soil dry mass (g) and T is the time (h) of sample incubation.

2.5 Microbial biomass

Microbial biomass was measured in soil samples from the third day of treatment using the substrate-induced respiration (SIR) method according to Mierzwa-Hersztek et al. (2018), . The SIR was determined by quantifying the evolution of carbon dioxide (72 h) after the addition of glucose ($200 \mu\text{g/g}$ of soil) in the same way as obtained by the basal respiration. Microbial biomass carbon (C_{mic}) was calculated according to the equation: $C_{mic}(\mu\text{g } C_{mic} \text{ g}^{-1} \text{ dry soil}) = 40.4R + 0.37$, where R is given in $\text{mg CO}_2 \text{ g}^{-1} \text{ h}^{-1}$.

2.6 Respiratory-Activation Quotient (QR)

The soil respiratory-activation quotient (QR) was obtained, according to Mierzwa-Hersztek et al. (2018), by calculating the ratio between the basal respiration (BSR) and substrate-induced respiration (SIR) for the same sample, ie $QR = BSR/SIR$.

2.7 Microbial metabolic activity

Fluorescein diacetate hydrolysis was performed according to Adam et al. (2001), with modifications to determine the esterase activity of the microbial population. Briefly, soil samples (0.1 g) were incubated in the presence of $750 \mu\text{L}$ of phosphate buffer (pH 7.6) and $50 \mu\text{L}$ of fluorescein diacetate at 30°C for 30 min. Then, $750 \mu\text{L}$ of chloroform/methanol (2:1) were added and the mixtures stirred. An Aliquot of the upper phase was removed after centrifugation at $2000 \times g$ for 3 min to be analyzed at 490 nm.

2.8 Statistical analysis

Data were evaluated for normality and homoscedasticity and analyzed by ANOVA. The means were tested by the F test and compared by the Scott-Knott test ($P < 0.05$), using the R program (version 4.2.1).

3. Results and discussion

3.1 Inhibitory activity of soil ureases

The amino acid derivatives D-Ac-106, D-106, L-106 and 116 were as efficient as the reference NBPT (Figure 2) as they inhibited ureases in the range from 57 - 75%. The remainder tested derivatives inhibited the enzyme by 2- 33%. The most used quality substances were selected to assess whether their presence in the environment is capable of promising the soil, which are generally used in the edaphic quality biomonitor, (Doran and Parkin, 1994). Biomonitoring corresponds to the response of living organisms to changes in their environment (Wittig, 1993).

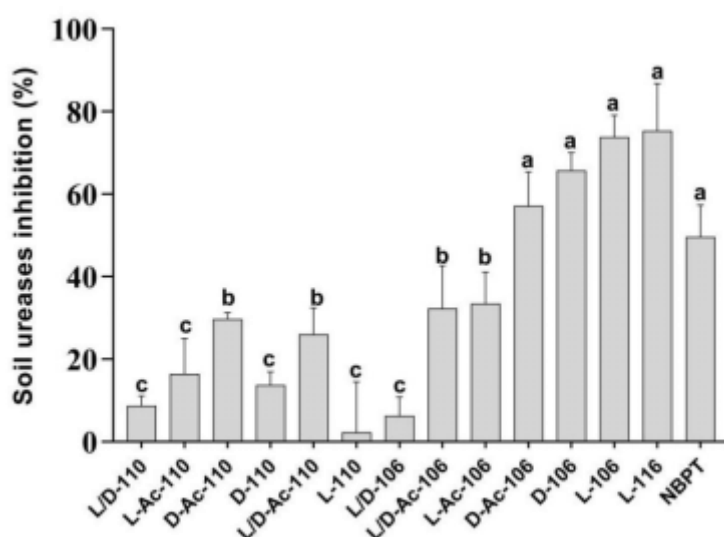


Figure 2. Effect of substances 110, 106, 116 and NBPT on the activity of ureases present in latossolo vermelho distrófico soil. Each substance was used at 500 μ M in the presence of 72 mM urea. Values correspond to the mean $\pm$ standard error of 3 independent experiments. Values followed by distinct letters are significantly different from each other by the Scott-Knott test ($P < 0.05$).

3.2 Microbial biomass

The microbial biomass obtained a higher value in soil with rhizospheric influence (Table 1). Root exudates (particularly from grasses root) released to the rhizosphere may benefit the microbiota, by increasing its biomass (Zhang et al., 2018; Tang et al.,

2020). Such exudates may include hormones, carbohydrates, amino acids, proteins, among others. Similar results were also found by Tang et al., (2020).

Microbial biomass is an important bioindicator of soil quality and is very sensitive to environment conditions (Balota et al., 2003). Possible effects of xenobiotics on the microbiota are promptly detected in a metabolically active soil (Beelen and Doelman, 1997). Microorganisms, despite representing a small fraction of the total organic matter, maintain soil structure and drive key ecosystem processes, such as decomposition and nutrient cycling (Basu et al., 2021). These processes, in turn, improve soil fertility and productivity (Singh and Gupta, 2018). Nitrogen fertilization has a variety of effects on the soil, such as positive (Liu et al., 2020), negative or even causes no changes in the microbiota (Lupwayi et al., 2012). Some studies have associated the increase in ammonium and urea content to lower microbial activity (Li et al., 2013). Application of urea at agronomically recommended rates did not affect microbial carbon biomass (MCB) in maize and barley (Lupwayi et al., 2012). Excessive application of urea has been associated with a decrease in the soil microbial biomass (Oliveira et al., 2021).

In the present work, application of urea in the soil (latossolo vermelho distrófico) in the presence or absence of urease inhibitors did not affect soil microbial biomass up to 35th days of experiment. The application of urea plus urease inhibitors promoted an increase in the microbial biomass up to the 7th day of treatment. At the end of the experiment, The microbial biomass was restored to the levels noted prior to fertilization.

Table 1. Microbin biomass ($\mu\text{g g}^{-1} \text{Cmic}$) in a dry sample of a Latossolo Vermelho distrófico, without the application of urea and with the application of urea with or without doses of different urease inhibitors, with or without the effect of millet rhizosphere.

Vessels with millet						
Treatment	1 st day before	3 rd day	7 th day	15 th day	35 th day	
Urea-free	142,7 Aa	124,4 Ba	144,5 Aa	143,9 Aa	151,8 Aa	
Inhibitor-free	143,6 Ab	198,5 Aa	202,7 Aa	150,5 Ab	139,9 Ab	
0,06% NBPT	140,3 Ab	197,8 Aa	205,9 Aa	144,8 Ab	142,9 Ab	
1% NBPT	154,7 Ab	186,6 Aa	209,3 Aa	156,4 Aa	155,2 Ab	
0,06 % D-106	146,2 Ab	193,0 Aa	223,1 Aa	148,4 Ab	151,3 Ab	
1% D-106	144,8 Ab	195,6 Aa	216,3 Aa	152,4 Ab	135,4 Ab	
0,06 % L-106	144,0 Ab	184,6 Aa	212,1 Aa	164,8 Ab	133,9 Ab	
1% L-106	142,5 Ab	189,6 Aa	229,4 Aa	160,4 Ab	144,6 Ab	
0,06 % L-116	146,8 Ab	175,4 Aa	241,0 Aa	138,4 Ab	141,8 Ab	
1% - L-116	140,8 Ab	171,8 Aa	198,9 Aa	149,6 Ab	134,6 Ab	
0,06% D-Ac-106	141,9 Ab	206,6 Aa	208,9 Aa	152,2 Ab	126,9 Ab	
1% D-Ac-106	150,7 Ab	169,5 Ab	207,1 Aa	168,4 Ab	134,7 Ab	
Vessels without millet						
Urea-free	90,8 Aa	87,5 Ba	92,1 Aa	97,7 Aa	92,9 Aa	

Vessels with millet										
Inhibitor-free	89,8	Ab	110,7	Aa	103,8	Aa	99,6	Ab	91,1	Ab
0,06% NBPT	88,0	Ab	108,4	Aa	111,6	Aa	98,5	Ab	93,2	Ab
1 % NBPT	92,3	Ab	103,7	Aa	105,3	Aa	99,1	Aa	90,6	Ab
0,06 % D-106	87,6	Ab	107,5	Aa	111,2	Aa	95,2	Ab	93,0	Ab
1% D-106	93,7	Ab	109,2	Aa	106,0	Aa	99,8	Ab	89,4	Ab
0,06 % L-106	82,1	Ab	106,7	Aa	108,9	Aa	97,1	Aa	90,2	Ab
1% L-106	95,8	Ab	99,1	Ab	109,3	Aa	97,7	Aa	95,9	Ab
0,06 % L-116	98,4	Ab	99,6	Aa	109,1	Aa	92,3	Ab	96,0	Ab
1% - L-116	87,3	Ab	107,9	Aa	107,3	Aa	97,7	Aa	93,6	Ab
0,06% D-Ac-106	95,6	Ab	103,6	Aa	107,6	Aa	93,9	Ab	95,0	Ab
1% D-Ac-106	91,9	Ab	104,1	Aa	103,5	Aa	96,8	Ab	92,0	Ab

Data correspond to the mean ($n = 5$). Values followed by distinct letters indicate significant difference among treatments in a due day while values followed by distinct lowercase letters denote significant difference for a treatment between the times of sample collections (Scott-Knott test; $P < 0.05$).

3.3 Respiratory-Activation Quotient (RQ)

The microbial respiration quotient relates the dormant microbial and the metabolically active community. This index can be used to assess the stability of microbial communities and, thus, disturbances in the edaphic ecosystem and possible toxic effects of xenobiotics. Thus, it is comparable to the analysis of qCO_2 (metabolic quotient; Anderson and Domsch, 1985). Metabolic quotient expresses the amount of CO_2 released per unit of microbial biomass and allows the identification of soils with better efficiency in the use of carbon and energy. The higher the qCO_2 values, the higher the environment disturbance or stress (Anderson and Domsch, 1993), as indicates low efficiency in the use of carbon and energy (Spohn et al., 2016). Toxic compounds tend to promote an increase in the ratio SBR/SIR), as well as qCO_2 , since they tend to suppress the increase in the metabolically active microorganisms population (Allegrini et al., 2017; Pal et al., 2006).

Maximum RQ was observed at the 3rd day of experiments in pots containing millet and at the 7th day in pots free of plants (table 2). The relatively higher proportion of metabolically active microbiota in the soils under rhizospheric influence is likely contributing to speeding up the use of carbon source added to the soil. These peaks are also related to the maximum increase respiration after urea addition (data not shown). The treatment with the inhibitors L-116 (1%) and D-Ac-106 (0.06%) increased RQ in soils containing millet after 7 days of treatment (Table 2), evidencing that the compounds somehow affected the microbiota at this period of time. In pots without

millet, however, L-116 (0.06% and 1%) and D-Ac-106 (0.06%) reduced these rates at the 3rd day of treatment due to the inhibition of the microbiota respiration.

Table 2. Effect of new urease inhibitors on the soil Respiratory-Activation Quotient.

Vessels with millet					
Treatment	1 st day before	3 rd day	7 th day	15 th day	35 th day
Urea-free	0,20 Aa	0,75 Aa	0,47 Ba	0,23 Aa	0,24 Aa
Inhibitor-free	0,16 Ab	0,97 Aa	0,33 Bb	0,23 Ab	0,25 Ab
0,06 % NBPT	0,23 Ab	0,55 Aa	0,33 Bb	0,27 Ab	0,27 Ab
1 % NBPT	0,19 Ab	0,67 Aa	0,40 Bb	0,28 Ab	0,24 Ab
0,06 % D-106	0,18 Ab	0,72 Aa	0,36 Bb	0,22 Ab	0,21 Ab
1 % D-106	0,17 Ab	0,72 Aa	0,37 Bb	0,26 Ab	0,29 Ab
0,06 % L-106	0,18 Ab	0,63 Aa	0,44 Ba	0,45 Aa	0,25 Ab
1% L-106	0,23 Ab	0,85 Aa	0,35 Bb	0,31 Ab	0,25 Ab
0,06 % L-116	0,20 Aa	0,61 Aa	0,42 Ba	0,39 Aa	0,25 Aa
1% - L-116	0,18 Ab	0,64 Aa	0,72 Aa	0,21 Ab	0,28 Ab
0,06% D-Ac-106	0,22 Ab	0,86 Aa	0,56 Aa	0,24 Ab	0,22 Ab
1% D-Ac-106	0,19 Ab	0,46 Aa	0,42 Ba	0,31 Ab	0,22 Ab
Vessels without millet					
Urea-free	0,30 Ac	0,66 Ab	1,47 Aa	0,38 Ac	0,35 Ac
Inhibitor-free	0,34 Ac	0,77 Ab	1,02 Aa	0,42 Ac	0,34 Ac
0,06 % - NBPT	0,40 Ac	0,70 Ab	1,16 Aa	0,28 Ac	0,32 Ac
1 % - NBPT	0,37 Ac	0,78 Ab	1,13 Aa	0,34 Ac	0,32 Ac
0,06 % - D-106	0,42 Ac	0,78 Ab	1,06 Aa	0,38 Ac	0,34 Ac
1 % - D-106	0,42 Ac	0,76 Ab	1,29 Aa	0,38 Ac	0,38 Ac
0,06 % - L-106	0,34 Ac	0,79 Ab	1,06 Aa	0,42 Ac	0,35 Ac
1% - L-106	0,28 Ac	0,72 Ab	1,16 Aa	0,32 Ac	0,34 Ac
0,06 % - L-116	0,33 Ac	0,62 Bb	1,15 Aa	0,48 Ac	0,38 Ac
1% - L-116	0,36 Ab	0,49 Bb	1,05 Aa	0,37 Ab	0,36 Ab
0,06% - D-Ac-106	0,39 Ac	0,53 Bb	1,16 Aa	0,40 Ac	0,30 Ac
1% - D-Ac-106	0,30 Ac	0,73 Ab	1,25 Aa	0,40 Ac	0,39 Ac

Data correspond to the mean (n = 5). Values followed by distinct capital letters indicate significant differences among treatments in a due day do not differ from each other, while values followed by distinct equal lowercase letters denote significant difference for a treatment between the times of sample collections (Scott-Knott test; P < 0.05).

3.4 Hydrolysis of fluorescein diacetate

The fluorescein diacetate hydrolysis (FDH) is an method to evaluate the total soil microbial activity related to the cleavage of ester bonds, since this hydrolysis is catalyzed by free enzymes such as lipases, proteases and esterases, as well as enzymes linked to biological membranes. For this reason, the FDH has been used to assess microbial activity in soil samples (Addam and Ducan, 2001). The greater the proportion of fluorescein released into the reaction medium, the higher the activity of soil microorganisms (Perucci et al., 1997). At the 7th day, the soil showed greater FDH

when treated with urea or urea plus urease inhibitors (Table 3). In pots containing millet, the compounds L-116 and D-Ac-106 at 1% stimulated the metabolism of the microbiota up to the 15th day. At the 3rd day, NBPT (1%) and the urease inhibitors D-106, L-106, L-116 and D-Ac-106 (1%) increased the FDH in soil devoid of plants when compared to urea treatment, a condition that persisted until the 7th day, except for NBPT and D-Ac-106 treatments. At the 35th day, there was no significant difference between treatments (Table 3). Contrasting results of FDH in soil have been reported regarding the use of xenobiotics. Herbicides, such as imazethapyr and flumioxazin did not affect the FDH by the microbiota of the dystrophic red oxisol (Pertile et al., 2020). On the other hand, sulfonylureas (herbicides) and pencycuron (pesticide) decreased the FDH in the microbiota of the dystrophic red oxisol (Sofa et al., 2012; Pal et al., 2006). Herein, NBPT and D-106 did not affect the FDA activity at the 7th day of treatment and apparently were not toxic to the microbiota of the dystrophic red oxisol as per the results of as RQ and microbial biomass. Complementary studies should be carried out to better understand the interaction of these compounds with the edaphic microbiota.

Table 3. Hydrolysis of fluorescein diacetate (μg fluorescein g^{-1} soil h^{-1}) in soil supplemented with urea and urease inhibitors.

Vessels with millet							
Treatment	1 st day before	1 st day	3 rd day	7 th day	15 th day	35 th day	
Urea-free	59,2 Aa	54,7 Aa	62,1 Aa	62,1 Ba	62,4 Ba	51,5 Aa	
Inhibitor-free	57,9 Ab	64,4 Ab	63,9 Ab	71,7 Aa	61,6 Bb	56,8 Ab	
0,06 % NBPT	56,6 Aa	63,3 Aa	58,5 Aa	62,1 Ba	62,8 Ba	54,2 Aa	
1 % NBPT	58,4 Aa	62,1 Aa	60,8 Aa	67,3 Aa	59,0 Ba	50,6 Aa	
0,06 % D-106	57,9 Aa	63,4 Aa	64,2 Aa	63,3 Ba	63,5 Ba	57,8 Aa	
1 % D-106	56,0 Aa	63,7 Aa	62,7 Aa	59,3 Ba	61,7 Ba	54,7 Aa	
0,06 % L-106	56,2 Aa	63,5 Aa	66,3 Aa	67,7 Aa	64,2 Ba	52,6 Aa	
1% L-106	57,2 Ab	65,5 Ab	68,6 Aa	71,3 Aa	65,1 Bb	59,3 Ab	
0,06 % L-116	54,3 Aa	65,4 Aa	58,4 Aa	67,8 Aa	63,5 Ba	55,4 Aa	
1% - L-116	54,1 Ab	66,1 Ab	62,3 Ab	71,8 Aa	70,7 Aa	61,6 Ab	
0,06% D-Ac-106	54,0 Ab	62,4 Ab	64,8 Ab	72,4 Aa	60,0 Bb	54,7 Ab	
1% D-Ac-106	53,3 Ab	63,7 Ab	59,3 Ab	76,2 Aa	70,3 Aa	55,9 Ab	
Vessels without millet							
Urea-free	53,5 Aa	55,3 Aa	50,3 Ba	66,3 Aa	55,5 Aa	49,0 Aa	
Inhibitor-free	56,4 Ab	54,5 Ab	54,5 Bb	66,1 Aa	62,0 Aa	55,3 Ab	
0,06 % NBPT	57,3 Aa	57,0 Aa	56,8 Ba	56,3 Ba	53,6 Aa	53,5 Aa	
1 % NBPT	51,9 Ab	58,5 Aa	61,1 Aa	63,0 Ba	58,0 Aa	48,5 Ab	
0,06 % D-106	51,2 Aa	57,0 Aa	57,2 Ba	60,0 Ba	60,4 Aa	58,5 Aa	
1 % D-106	55,5 Aa	64,4 Aa	69,1 Aa	61,9 Ba	63,6 Aa	58,7 Aa	
0,06 % L-106	57,4 Aa	59,9 Aa	60,7 Aa	65,2 Aa	62,7 Aa	49,9 Aa	
1% L-106	52,6 Aa	63,5 Aa	67,3 Aa	66,9 Aa	60,6 Aa	54,4 Aa	

0,06 % L-116	58,5	Aa	61,0	Aa	61,2	Aa	67,5	Aa	65,0	Aa	53,7	Aa
1% - L-116	53,0	Aa	63,3	Aa	65,3	Aa	69,8	Aa	63,1	Aa	57,6	Aa
0,06% D-Ac-106	57,2	Aa	62,3	Aa	61,7	Aa	73,2	Aa	68,4	Aa	48,1	Aa
1% D-Ac-106	54,9	Aa	59,0	Aa	67,5	Aa	73,3	Aa	62,2	Aa	56,8	Aa

Data correspond to the mean (n = 5). Values followed by distinct capital letters indicate significant differences among treatments in a due day do not differ from each other, while values followed by distinct equal lowercase letters denote significant difference for a treatment between the times of sample collections (Scott-Knott test; $P < 0.05$).

3.5 Effect of new urease inhibitors on millet development grown under controlled condition

3.5.1 Plant biomass accumulation

Nitrogen fertilization stimulated the development of shoot and roots and the presence of the inhibitors did not affect the shoot development (Figure 3). The presence of D-Ac-106 (1%) stimulated the growth of roots, which resulted in a 65% increase in the roots biomass. Biostimulants refer to the molecules capable of improving plant development and growth by boosting the efficiency in the use of nutrients, the tolerance to abiotic stress, and consequently crop yields (Mutale-Joan et al., 2020). Thus, the compound D-Ac-106 constitutes a promising biostimulant to be further investigated as an additive in fertilizers formulations.

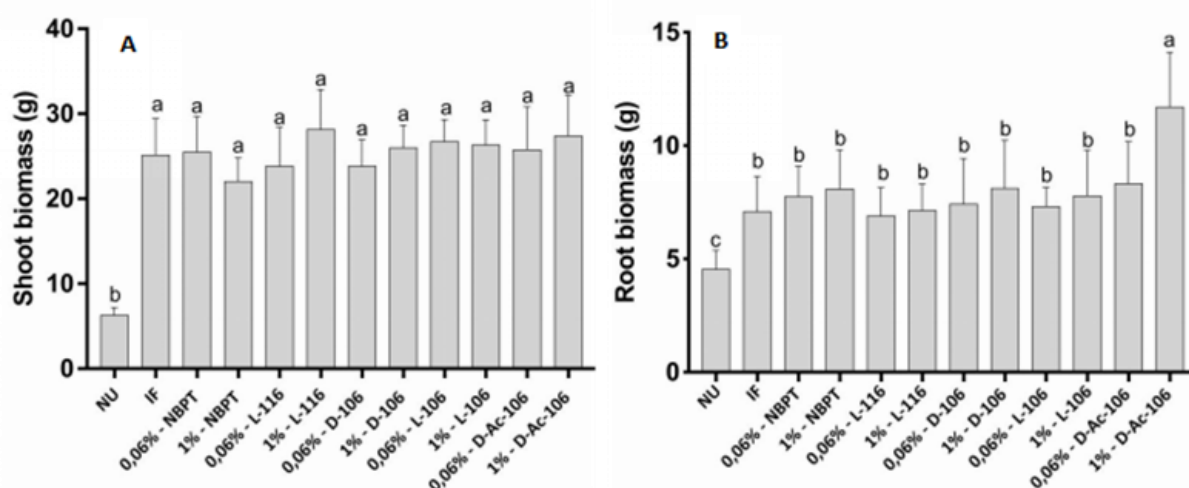


Figure 2. Effect of new urease inhibitors on biomass accumulation in shoot (A) and root system (B). Values correspond to the mean $\pm$ standard deviation (n=5). Values followed by distinct letters are significantly different from each other by the Scott-Knott test ($P < 0.05$).

The NBPT and D-Ac-106 at 1% stimulated the allocation of resources in the root system, evidencing their positive effect on millet metabolism. Lower shoot/root ratio obtained for plants grown in the presence of D-Ac-106 0.06% is due to the stimulation of biomass accumulation in roots. However, decrease in millet height (Table 4) and shoot biomass (Figure 3) was not evidenced with treatment.

Table 4. Effect of new urease inhibitors on the development.

Treatment	Height (cm)	RWC (Shoot) (g)	Shoot/root biomass (g)
Urea-free	21,1 ± 4,9 b	27,7 ± 2,6 b	1,4 ± 0,2 c
Inhibitor-freeF	84,1 ± 32,3 a	110,3 ± 12,7 a	3,7 ± 1,0 a
0,06 % NBPT	93,1 ± 30,3 a	110,4 ± 15,5 a	3,4 ± 0,8 a
1 % NBPT	66,0 ± 20,2 a	103,6 ± 6,3 a	2,8 ± 0,4 b
0,06 % D-106	78,9 ± 22,6 a	119,4 ± 22,4 a	3,6 ± 0,9 a
1 % D-106	90,7 ± 28,2 a	125,2 ± 11,2 a	4,0 ± 0,8 a
0,06 % L-106	84,3 ± 22,4 a	113,0 ± 14,9 a	3,4 ± 1,0 a
1% L-106	82,7 ± 21,0 a	117,2 ± 7,9 a	3,4 ± 0,9 a
0,06 % L-116	83,0 ± 15,5 a	124,7 ± 14,8 a	3,7 ± 0,3 a
1% - L-116	77,3 ± 8,7 a	111,2 ± 6,7 a	3,5 ± 0,9 a
0,06% D-Ac-106	82,8 ± 30,8 a	113,9 ± 16,6 a	3,3 ± 1,2 a
1% D-Ac-106	77,0 ± 21,6 a	116,6 ± 14,0 a	2,4 ± 0,4 b

Data correspond to the mean ± standard deviation (n = 5). Distinct letters indicate significant differences among the treatments (P < 0.05; Scott-Knott test). RWC (Relative Water Content).

3.5.2 Chloroplast pigments

Treatment with urea solely promoted a 100% increase in chlorophyll production in comparison to plants under urea-free treatment (Table 5). Treatment with inhibitors did not affect the content of chloroplast pigments.

Table 5. Effect of urease inhibitors on chloroplast pigments (mg.g⁻¹ FW).

Treatment	chlorophyll a	chlorophyll b	Total chlorophyll	Chlorophyll a/b	Carotenoids
Urea-free	0,27 ± 0,1 b	0,15 ± 0,0 b	0,69 ± 0,1 b	1,76 ± 0,2 a	0,13 ± 0,0 b
Inhibitor-free	0,57 ± 0,1 a	0,27 ± 0,0 a	1,29 ± 0,2 a	2,11 ± 0,1 a	0,24 ± 0,0 a
0,06 % NBPT	0,64 ± 0,2 a	0,29 ± 0,1 a	1,43 ± 0,3 a	2,22 ± 0,2 a	0,27 ± 0,1 a
1 % NBPT	0,69 ± 0,1 a	0,30 ± 0,0 a	1,51 ± 0,2 a	2,26 ± 0,1 a	0,28 ± 0,0 a
0,06 % D-106	0,69 ± 0,2 a	0,31 ± 0,0 a	1,49 ± 0,2 a	2,55 ± 1,2 a	0,27 ± 0,0 a
1 % D-106	0,69 ± 0,1 a	0,33 ± 0,1 a	1,57 ± 0,1 a	2,87 ± 2,3 a	0,29 ± 0,0 a
0,06 % L-106	0,71 ± 0,1 a	0,32 ± 0,0 a	1,53 ± 0,2 a	2,20 ± 0,1 a	0,28 ± 0,0 a
1% L-106	0,79 ± 0,1 a	0,35 ± 0,0 a	1,69 ± 0,2 a	2,26 ± 0,0 a	0,31 ± 0,0 a
0,06 % L-116	0,79 ± 0,1 a	0,34 ± 0,0 a	1,68 ± 0,3 a	2,29 ± 0,1 a	0,31 ± 0,0 a
1% - L-116	0,63 ± 0,1 a	0,29 ± 0,0 a	1,39 ± 0,1 a	2,20 ± 0,1 a	0,26 ± 0,0 a
0,06% D-Ac-106	0,70 ± 0,1 a	0,31 ± 0,0 a	1,54 ± 0,2 a	2,24 ± 0,1 a	0,28 ± 0,0 a
1% D-Ac-106	0,61 ± 0,1 a	0,27 ± 0,0 a	1,36 ± 0,2 a	2,24 ± 0,1 a	0,25 ± 0,0 a

Data correspond to the mean ± standard deviation (n = 5). Distinct letters indicate significant differences among the treatments (P < 0.05; Scott-Knott test).

4. Conclusions

Soil changes may imbalance microbiota activity and consequently the geobiochemical nutrients cycle. Therefore, the absence of a negative effect of urease inhibitors (at the tested concentrations) on the soil microbiota suggests that such organic substances do not compromise the soil quality. Additionally, none of the urease inhibitors based on amino acid derivatives impaired the millet development in vessels. On the contrary, the urease inhibitor D-Ac-106 acted as a biostimulant concerning the roots growth. Such findings reveal the great potential of D-Ac-106 as an additive in urea-based formulations for the ability to stimulate root growth and thus a better exploitation of the soil resources (water and nutrients) by roots.

References

- Abdalla, Z. F., El-Sawy, S., El-Bassiony, A. E. M., Jun, H., Shedeed, S., Okasha, A. M., & Prokisch, J., 2022. Smart Fertilizers vs. Nano-fertilizers: A Pictorial Overview. *Environment, Biodiversity and Soil Security*. 6, 191-204. <https://doi.org/10.21608/JENVBS.2022.153990.1184>
- Adam, G., & Duncan, H., 2001. Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. *Soil biology and biochemistry*. 33, 943-951. [https://doi.org/10.1016/S0038-0717\(00\)00244-3](https://doi.org/10.1016/S0038-0717(00)00244-3)
- Alef, K., Nannipieri, P., 1995. Enrichment, isolation and counting of soil microorganisms, in: *Methods in Applied Soil Microbiology and Biochemistry*. Elsevier, pp. 123–191. <https://doi.org/10.1016/B978-012513840-6/50019-7>
- Allegrini, M., Gomez, E. D. V., & Zabaloy, M. C., 2017. Repeated glyphosate exposure induces shifts in nitrifying communities and metabolism of phenylpropanoids. *Soil Biology and Biochemistry*. 105, 206-215. <https://doi.org/10.1016/j.soilbio.2016.11.024>
- Anderson, J.P.E. and Domsch, K.H., 1978. A Physiological Method for the Quantitative Measurement of Microbial Biomass in Soil. *Soil Biology and Biochemistry*. 11, 215-221. [http://dx.doi.org/10.1016/0038-0717\(78\)90099-8](http://dx.doi.org/10.1016/0038-0717(78)90099-8)
- Anderson, T.H. and Domsch, K.H., 1993. The metabolic quotient for CO₂ (qCO₂) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils. *Soil Biology & Biochemistry*. 25, 393-395. [http://dx.doi.org/10.1016/0038-0717\(93\)90140-7](http://dx.doi.org/10.1016/0038-0717(93)90140-7)
- Araújo, D. P., Morais, S. V. S. S., de Fátima, A., Modolo, L. V., 2015. Efficient sodium bisulfite-catalyzed synthesis of benzothiazoles and their potential as ureases inhibitors. *RSC Advances: an international journal to further the chemical sciences*. 5, 28814-28821. <https://doi.org/10.1039/C5RA01081K>
- Balota, E. L., Andrade, D. S., Colozzi Filho, A., Dick, R. P., 2003. Microbial biomass in soils under different tillage and crop rotation systems. *Biology and Fertility of Soils*. 38, 15-20. <https://doi.org/10.1007/s00374-003-0590-9>
- Basu, S., Kumar, G., Chhabra, S., & Prasad, R., 2021. Role of soil microbes in biogeochemical cycle for enhancing soil fertility. In *New and Future Developments in Microbial Biotechnology and Bioengineering*. Elsevier, pp. 149-157.

- <https://doi.org/10.1016/B978-0-444-64325-4.00013-4>
- Beelen, P.V. and Doelman, P., 1997. Significance and Application of Microbial Toxicity Tests in Assessing Ecotoxicological Risks of Contaminants in Soil and Sediment. *Chemosphere*. 34, 455-499. [https://doi.org/10.1016/S0045-6535\(96\)00388-8](https://doi.org/10.1016/S0045-6535(96)00388-8)
- Beretta-Blanco, A., Pérez, O., & Carrasco-Letelier, L., 2019. Soil quality decrease over 13 years of agricultural production. *Nutrient Cycling in Agroecosystems*, 114(1), 45-55. <https://doi.org/10.1007/s10705-019-09990-3>
- Brito, T. O., Souza, A. X., Mota, Y. C. C., Morais, V. S. S., Souza, L. T., de Fátima, A., Macedo Jr., F. C., Modolo, L. V., 2015. Design, syntheses and evaluation of benzoylthioureas as urease inhibitors of agricultural interest. *RSC Advances: an international journal to further the chemical sciences*. 5, 44507-44515. <https://doi.org/10.1039/C5RA07886E>
- Camargo, P. G., Fabris, M., Nakamae, M. Y. T., de Freitas Oliveira, B. G., da Silva Lima, C. H., de Fátima, Â., ... & Macedo Jr, F., 2022. (thio)hydantoins and hydantoins derived from amino acids as potent urease inhibitors: Inhibitory activity and ligand-target interactions. *Chemico-Biological Interactions*, 365, 110045. <https://doi.org/10.1016/j.cbi.2022.110045>
- Chen, Y., Chu, C., He, F., & Fang, S., 2022. A mechanistic model for nitrogen-limited plant growth. *Annals of Botany*. 129, 583-592. <https://doi.org/10.1093/aob/mcac018>
- Doran, J. W., Parkin, T. B., 1994. Defining and assessing soil quality. In: DORAN, J.W., Coleman, D.C., Bezdicsek, D. F., Stewart, B. A.(Org.) *Defining soil quality for a sustainable environment*. Madison: SSSA, pp. 3-21. <https://doi.org/10.2136/sssaspecpub35.c1>
- Engel, R. E., Towey, B. D., & Gravens, E., 2015. Degradation of the Urease Inhibitor NBPT as Affected by Soil pH. *Soil Science Society of America Journal*. 79, 1674. <https://doi.org/10.2136/sssaj2015.05.0169>
- Fan, X., Yin, C., Yan, G., Cui, P., Shen, Q., Wang, Q., & Liang, Y., 2018. The contrasting effects of N-(n-butyl) thiophosphoric triamide (NBPT) on N₂O emissions in arable soils differing in pH are underlain by complex microbial mechanisms. *Science of the total environment*. 642, 155-167. <https://doi.org/10.1016/j.scitotenv.2018.05.356>
- Gianfreda, L., Rao M.A., 2017. Soil microbial and enzymatic diversity as affected by the presence of xenobiotics. In: Hashmi M., Kumar V., Varma A. (eds) *Xenobiotics*

- in the Soil Environment. *Soil Biology*. Springer, Cham, pp 153-19.
https://doi.org/10.1007/978-3-319-47744-2_12
- Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol Fertil Soils*. 6, 68-72.
<https://doi.org/10.1007/BF00257924>
- Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol*. 148, 350–382. [https://doi.org/10.1016/0076-6879\(87\)48036-1](https://doi.org/10.1016/0076-6879(87)48036-1)
- Li, F., Liu, M., Li, Z., Jiang, C., Han, F., & Che, Y., 2013. Changes in soil microbial biomass and functional diversity with a nitrogen gradient in soil columns. *Appl Soil Ecol*. 64, 1-6. <https://doi.org/10.1016/j.apsoil.2012.10.006>
- Liu, Q., Liu, B., Zhang, Y., Hu, T., Lin, Z., Liu, G., ... & Xie, Z., 2019. Biochar application as a tool to decrease soil nitrogen losses (NH₃ volatilization, N₂O emissions, and N leaching) from croplands: Options and mitigation strength in a global perspective. *Global change biology*. 25, 2077-2093.
<https://doi.org/10.1111/gcb.14613>
- Liu, C., Gong, X., Dang, K., Li, J., Yang, P., Gao, X., ... Feng, B., 2020. Linkages between nutrient ratio and the microbial community in rhizosphere soil following fertilizer management. *Environ. Res*. 184, 109261.
<https://doi.org/10.1016/j.envres.2020.109261>
- Lupwayi, N. Z., Lafond, G. P., Ziadi, N., & Grant, C. A., 2012. Soil microbial response to nitrogen fertilizer and tillage in barley and corn. *Soil and Tillage Research*. 118, 139-146. <https://doi.org/10.1016/j.still.2011.11.006>
- Mierzwa-Hersztek, M., Klimkowicz-Pawlas, A., & Gondek, K., 2018. Influence of poultry litter and poultry litter biochar on soil microbial respiration and nitrifying bacteria activity. *Waste and biomass valorization*. 9(3), 379-389.
<https://doi.org/10.1007/s12649-017-0013-z>
- Modolo, L.V, Da-Silva, C.J., Brandão, D.S., Chaves, I.S., 2018. A minireview on what we have learned about urease inhibitors of agricultural interest since mid-2000s. *Journal of Advanced Research*. 13, 29-37.
<https://doi.org/10.1016/j.jare.2018.04.001>
- Mutale-Joan, C., Redouane, B., Najib, E., Yassine, K., Lyamlouli, K., Laila, S., ... & Hicham, E. A. 2020. Screening of microalgae liquid extracts for their bio stimulant properties on plant growth, nutrient uptake and metabolite profile of *Solanum*

- lycopersicum L. Scientific reports, 10(1), 1-12. | <https://doi.org/10.1038/s41598-020-59840-4>
- Odake, S., Nakahashi, K., Morikawa, T., Takebe, S., Kobashi, K., 1992. Inhibition of Urease Activity by Dipeptidyl Hydroxamic Acids. Chem. Pharm. Bull. 40, 2764–2768. <https://doi.org/10.1248/cpb.40.2764>
- Oliveira, F. M., Barbosa, L. C. A., Demuner, A. J., Maltha, C. R. A., Pereira, S. R., Horta, L. P., Modolo, L. V., 2014. Synthesis, molecular properties and DFT studies of new phosphoramidates as potential urease inhibitors. Medicinal Chemistry Research. 23, 5174-5187. <https://doi.org/10.1007/s00044-014-1113-y>
- Oliveira, V. A., Brasil, E. P. F., Teixeira, W. G., dos Santos, F. C. V., & da Silva, A. R., 2021. Effects of Enhanced-Efficiency Nitrogen Fertilizers on Soil Microbial Biomass and Respiration in Tropical Soil Under Upland Rice Cultivation. Journal of Agricultural Science, 13. <https://doi.org/10.5539/jas.v13n6p31>
- Perucci, P., Bonciarelli, U., Santilocchi, R., & Bianchi, A. A., 1997. Effect of rotation, nitrogen fertilization and management of crop residues on some chemical, microbiological and biochemical properties of soil. Biology and fertility of soils. 24, 311-316. <https://doi.org/10.1007/s003740050249>
- Pertile, M., Antunes, J. E. L., Araujo, F. F., Mendes, L. W., Van den Brink, P. J., & Araujo, A. S. F. (2020). Responses of soil microbial biomass and enzyme activity to herbicides imazethapyr and flumioxazin. Scientific Reports, 10(1), 1-9. <https://doi.org/10.1038/s41598-020-64648-3>
- Singh, J. S., & Gupta, V. K., 2018. Soil microbial biomass: A key soil driver in management of ecosystem functioning. Science of the Total Environment. 634, 497-500. <https://doi.org/10.1016/j.scitotenv.2018.03.373>
- Sofo, A., Scopa, A., Dumontet, S., Mazzatura, A., & Pasquale, V., 2012. Toxic effects of four sulphonylureas herbicides on soil microbial biomass. Journal of Environmental Science and Health, Part B. 47(7), 653-659. <https://doi.org/10.1080/03601234.2012.669205>
- Spohn, M., Klaus, K., Wanek, W., & Richter, A., 2016. Microbial carbon use efficiency and biomass turnover times depending on soil depth—Implications for carbon cycling. Soil Biology and Biochemistry. 96, 74-81. <https://doi.org/10.1016/j.soilbio.2016.01.016>
- Trenkel, M.E., 2010. Slow and Controlled Release and Stabilized Fertilizers: An Option for Enhancing Nutrient Use Efficiency in Agriculture. Paris: International Fertilizer

Industry Association, 167.

Ullah, A., Iftikhar, F., Arfan, M., Batool Kazmi, S.T., Anjum, M.N., Haq, I., Ayaz, M., Farooq, S., Rashid, U., 2018. Amino acid conjugated antimicrobial drugs: Synthesis, lipophilicity- activity relationship, antibacterial and urease inhibition activity. *Eur. J. Med. Chem.* 145, 140–153.

<https://doi.org/10.1016/j.ejmech.2017.12.089>

Watson, C.J., Akhonzada, N.A., Hamilton, J.T.G., Matthews, D.I., 2008. Rate and mode of application of the urease inhibitor N-(n-butyl) thiophosphoric triamide on ammonia volatilization from surface-applied urea. *Soil Use Manag.* 24, 246–253.

<https://doi.org/10.1111/j.1475-2743.2008.00157.x>

Wittig, R., 1993. General aspects of biomonitoring heavy metals by plants. In: MARKERT, B. (Org.) *Plant as biomonitors. Indicators for heavy metals in the terrestrial environment.* Weinheim: VCH, p. 3-2.

<https://doi.org/10.1002/pca.2800060209>

Zhang, C., Liu, G., Xue, S., Wang, G., Wang, J., & Song, Z., 2018. Effects of rhizosphere interactions of grass interspecies on the soil microbial properties during the natural succession in the Loess Plateau. *European Journal of Soil Biology.* 85, 79-88. <https://doi.org/10.1016/j.ejsobi.2018.01.006>

Chapter 2

New urease inhibitors improve initial root development and do not affect the enzymatic antioxidant metabolism in Pearl Millet

Abstract

Urease is an enzyme present in soil that speeds up the hydrolysis of urea by over 1 billion times. This rapid hydrolysis on the soil surface is the cause of most of the nitrogen loss from urea due to the volatilization of the ammonia formed in the catalysis process. Substances that inhibit soil ureases have been extensively researched to be used in technologies that focus on the improvement of fertilizers efficiency. For this purpose, a promising urease inhibitor should not exhibit toxicity to soil microbiota and plants. The seed germination is a process widely used to address the potential toxicity of a compound toward plants. This work investigated the effect of the novel urease inhibitors D-tryptophan derivatives, D-Ac-106 and D-106, on seed germination, early plant development and some enzymes from the antioxidant system of *Cenchrus americanus* (millet). No apparent negative effect on the initial development of pearl millet was observed when millet seeds were challenged with D-Ac-106 or D-106 at concentrations in the range from 25 to 100 μ M. Notably, both urease inhibitors stimulated the growth of the millet primary roots. Furthermore, these compounds appear to be apparently safe for plants, as they do not stimulate antioxidant metabolism enzymes. Thus, (Thio)hydantoins derived from the amino acid D-tryptophan exert a very interesting beneficial effect since the better development of the root system can bring improvements to the plants by better exploiting the soil and acquiring water and nutrients.

Keywords: *Cenchrus americanus*, phytotoxicity, Plant development, urea fertilization, Urease inhibitors.

1. Introduction

The use of nitrogen fertilizers is essential to enable high yields (Chen et al., 2022). Urea is the nitrogen fertilizer most used worldwide in agriculture. However, its efficiency is around 50%, due to N losses derived from nitrate leaching and/or ammonia volatilization (Liu et al., 2019). This is because soil ureases can catalyze the hydrolysis of urea to form the gasses carbon dioxide and ammonia. Ammonia can either go to the atmosphere or be oxidized to nitrate, which in turn may be leached from soil. Fertilizers of increased efficiency based on the use of inhibitors of a variety of reactions (urea hydrolysis or ammonia oxidation away from the rhizosphere) have emerged to overcome N losses (Trenkel, 2010). The use of urease inhibitors is among these technologies aiming to improve urease efficiency. This work investigated the effect of novel urease inhibitors derived from amino acids core on the germination and early development of *Cenchrus americanus* (millet). Despite being originated from amino acids, substances essential to plant metabolism, one cannot rule out the possibility of such derivatives being toxic. For instance, L-canavanine, an L-arginine analogue, is naturally produced by *Canavalia ensiformis* plants to trick herbivores to produce non-functional proteins since L-canavanine is incorporated into the peptide chains during the protein biosynthesis (Rosenthal, 1991). Furthermore, the new compounds evaluated are derived from D-tryptophan. Dextrogenous amino acids are known to be toxic to various organisms (Zhang and Sun, 2014). However, information about the effects of D-amino acids on plants is very rare. Some studies demonstrate that D-amino acids in plants are physiologically active compounds. Chloroplast division and adaptation of nitrogen metabolism to stress exposure are some evidence of the effects of D-amino acids in plants (Kolukisaoglu and Suarez, 2017).

The compounds evaluated in this work are derived substances and the fact that some substances may bear oxidant core(s), this can cause the formation of free radicals that are detrimental to cell functioning. (Sharma and Dubey, 2012). Antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) contribute to the plant response to stress (Mittler., 2002). They protect both cells and plant subcellular compartments from the cytotoxic effects of free radicals and reactive species and influence several reactions involved in defense signaling (Soares, 2007). In addition to altering antioxidant metabolism, xenobiotics, alone or in

combination with other molecules, can impair the growth and physiology of organisms (Singh et al., 2016).

Thus, the present work seeks to evaluate the effect of on the initial development of millet of potential substances to be used in agriculture. In addition, it brings new evidence on the physiological effect of D-amino acid derivatives on plants.

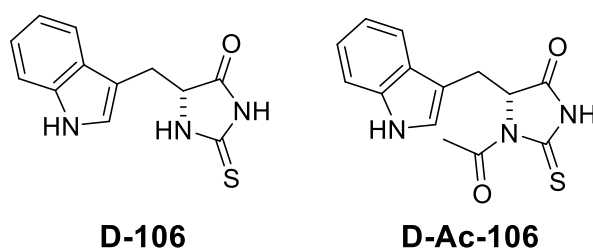


Figure 1. Structures of acetyl (thio)hydantoin and thiohydantoin.

2. Material and methods

2.1 Initial development of millet seedlings

Germination tests were carried out using millet seeds (cultivar BR1501) in GERBOX® plates containing a double sheet of filter paper moistened with a 0.5% Nystatin solution (Lemos Filho et al., 1997). Seeds that show root protrusion were considered germinated (Bewley and Black, 1994). The experimental design was completely randomized in a factorial scheme with five replications of 30 seeds per plate. The urease inhibitors were tested at 25, 50 and 100 μM . The organic substances were dissolved in a solution containing (0.025 %) ethanol, (0.025 %) DMSO, (0.25 %) propylene glycol and (0.7 %) polyethylene glycol 400. A control containing only the pure diluent solution without the addition of inhibitors was evaluated. The plates were maintained under white fluorescent light ($30 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) at 25 °C, with a photoperiod of 12 h. Germination counting was performed daily. After 7 days, the following indexes were evaluated: germination percentage (G), Germination Speed Index (IVG), average germination speed (VMG) and average germination time of 50% of the seeds (T50) (Carvalho and Carvalho, 2009; Soltani et al., 2015). The root length was measured using the ImageJ program and the biomass accumulation was obtained based on the weight of the seedlings dried in an oven at 55°C. The activity of antioxidant enzymes was also determined in 7-old seedlings that originated from millet seeds treated with the urease inhibitors for 7 days.

2.2 Antioxidant metabolism enzyme activity

The enzymatic extract was obtained by grinding the seedlings in liquid nitrogen followed by centrifugation at $12,000 \times g$ for 30 min. at 4 °C. The resulting supernatants were used in the enzymatic assay to determine the activity of the superoxide dismutase, catalase, and ascorbate peroxidase. The quantification of total proteins was performed using the method of Bradford (1976) and bovine serum albumin (BSA) as a standard.

The catalase (CAT) activity was determined according to da-Silva et al., (2017). The samples were incubated in a reaction medium consisting of 50 mM phosphate buffer (pH 6.8) and 125 μM H_2O_2 . The enzyme activity was estimated by measuring

the absorbance at 240 nm to monitor H₂O₂ degradation and using the molar extinction coefficient of 39.4 mM⁻¹ cm⁻¹.

The ascorbate peroxidase (APX) activity was determined according to Nakano and Asada (1981), by monitoring the rate of ascorbate oxidation at 290 nm. The test buffer was composed of 50 mM phosphate buffer (pH 6.0), 1 mM ascorbic acid and 2 mM H₂O₂. The molar extinction coefficient of 2.8 L mmol⁻¹ cm⁻¹ was used to determine the APX activity.

The superoxide dismutase (SOD) activity was evaluated according to (Giannopolitis and Ries, 1977), with modifications (Horta et al., 2014). Briefly, the reaction medium consisted of 50 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 75 µM nitrotetrazolium blue (NBT), 100 µM EDTA and 2 µM riboflavin. Reactions were set in the presence or absence of seedling extract and in the presence or absence of light (15 W for 10 min) at room temperature. The quantification of SOD activity occurred by monitoring the formation of blue formazan at 575 nm.

2.3 Statistical analysis

Data were evaluated using analysis of variance (ANOVA). Means were tested using the F test and compared using the Scott-Knott test ($P < 0.05$) using the R program (4.2.1 version). The activity of the antioxidant enzymes from treated samples was compared with those of control seedlings using one-way-anova. The normality and homoscedasticity of the data were evaluated by the Shapiro-Wilk and Brown-Forsythe test, respectively.

3. Results and discussion

3.1 Germination and initial development of millet

The compounds D-Ac-106, D-106 and NBPT did not affect any of the germination parameters evaluated during the seven days of experiment regardless of the concentration (Table 1). This suggests a lack of toxicity by the compounds at concentrations tested. The seed germination process is constituted of three stages, namely imbibition (phase I), root emergence (phase II) and root elongation (phase III) (Luo et al., 2018). The seed coat has been reported as a physical barrier for xenobiotics, which may prevent the contact of the foreign substance with the embryo (Zhang et al., 2015). Such protection may be taking place in millet seeds challenged with the urease inhibitors since the seeds of such plant species hold a rigid coat adhered to a fragile endosperm (Rathore & Singh, 2017). None of the compounds impaired the root emergence (phase II), even after the rupture of the tegument. Some studies suggest that compounds that inhibit seed germination might be considered to be more toxic than those that inhibit root elongation (Luo et al., 2018). Surprisingly, the treatment of millet seeds with D-Ac-106 or D-106 improved the growth of primary roots compared to the control treatment (Figure 2 and Table 2). Such amino acid derivatives functioned as biostimulants as they did toward lettuce seeds (Chapter 1). The use of biostimulants in agriculture is useful as some substances, such as some triazoles, improve plant performance and productivity besides providing greater tolerance against abiotic stresses (Desta and Amare, 2021). Substances that promote root development are desired for ensuring better soil exploitation by plants, and increasing the possibility of establishing symbiotic relationships such as with mycorrhizae.

A number of agrochemicals and pollutants have been shown to alter the oxidative balance in plants (Parween et al., 2016). This can promote intracellular increase of reactive oxygen species (ROS) that react with macromolecules promoting and causing metabolic derangements. The increase in the ROS levels is usually accompanied by an increase in the activity of the enzymes catalase, ascorbate peroxidase and superoxide dismutase. These enzymes eliminate excess ROS to restore ROS homeostasis. Notably, none of the compounds at the concentrations tested stimulated the enzymatic antioxidant system in millet seedlings, suggesting that the contact between each urease inhibitor with millet seeds for 7 days did not trigger an oxidative stress in the seedlings (Figure 3).

Table 1. Effect of new urease inhibitors on germination.

Treatment	Concentration (μM)	G (%)	IVG	VMG (dias)	T50 (dias)
Control	-	87,8 $\pm$ 3,3 a	20,4 $\pm$ 0,5 a	0,70 $\pm$ 0,0 a	0,77 $\pm$ 0,1 a
NBPT	25	84,9 $\pm$ 12,4 a	18,3 $\pm$ 1,6 a	0,66 $\pm$ 0,1 a	0,79 $\pm$ 0,1 a
	50	88,1 $\pm$ 9,3 a	20,0 $\pm$ 1,0 a	0,77 $\pm$ 0,1 a	0,79 $\pm$ 0,1 a
	100	95,5 $\pm$ 5,1 a	21,4 $\pm$ 2,4 a	0,75 $\pm$ 0,1 a	0,72 $\pm$ 0,1 a
D-106	25	90,7 $\pm$ 3,2 a	21,1 $\pm$ 1,4 a	0,73 $\pm$ 0,1 a	0,68 $\pm$ 0,2 a
	50	91,4 $\pm$ 5,4 a	22,1 $\pm$ 1,6 a	0,76 $\pm$ 0,0 a	0,70 $\pm$ 0,1 a
	100	91,2 $\pm$ 7,7 a	21,0 $\pm$ 2,2 a	0,75 $\pm$ 0,0 a	0,68 $\pm$ 0,1 a
106-AcD	25	88,4 $\pm$ 6,9 a	21,2 $\pm$ 2,8 a	0,75 $\pm$ 0,1 a	0,66 $\pm$ 0,1 a
	50	88,1 $\pm$ 7,1 a	21,6 $\pm$ 2,9 a	0,80 $\pm$ 0,1 a	0,63 $\pm$ 0,1 a
	100	85,2 $\pm$ 9,4 a	20,3 $\pm$ 3,0 a	0,84 $\pm$ 0,0 a	0,65 $\pm$ 0,1 a

Data correspond to the mean $\pm$ standard deviation (n = 5). Distinct letters indicate significant differences among the treatments (P < 0.05; Scott-Knott test).



Figure 2. Pearl Millet seedling originated from seeds that germinated in the presence of D-106 (100 μM) or D-Ac-106 (100 μM) for 7 days.

Table 2. Effect of urease inhibitors on the early stages of millet development.

Treatment	Concentration (μM)	Root lenght (mm)	Biomass (g)
Control	-	915,9 $\pm$ 62,8 c	0,11 $\pm$ 0,3 a
NBPT	25	1114,1 $\pm$ 204,2 c	0,11 $\pm$ 0,0 a
	50	1371,0 $\pm$ 263,4 b	0,10 $\pm$ 0,0 a
	100	1123,7 $\pm$ 265,8 c	0,09 $\pm$ 0,0 a
D-106	25	1389,0 $\pm$ 188,9 b	0,10 $\pm$ 0,0 a
	50	1727,0 $\pm$ 151,1 a	0,09 $\pm$ 0,0 a
	100	1659,0 $\pm$ 217,2 a	0,10 $\pm$ 0,0 a
D-Ac-106	25	1555,1 $\pm$ 129,2 a	0,09 $\pm$ 0,0 a
	50	1660,0 $\pm$ 123,2 a	0,10 $\pm$ 0,0 a
	100	1770,0 $\pm$ 146,0 a	0,10 $\pm$ 0,0 a

Data correspond to the mean $\pm$ standard deviation (n = 5). Distinct letters indicate significant differences among the treatments (P < 0.05; Scott-Knott test).

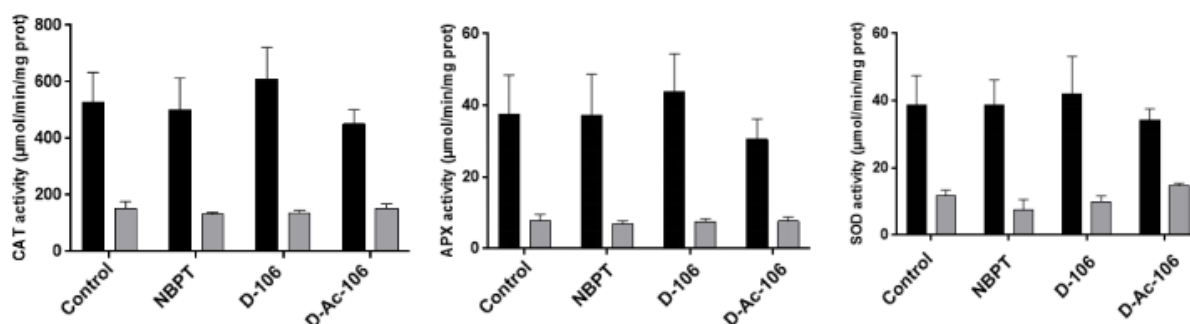


Figure 3. Effect of D-106 and D-Ac-106 on the activity of antioxidant enzymes of millet. Data correspond to the mean $\pm$ standard deviation (n = 3). The means of results for shoot (black bars) and roots (gray bars) were compared to each other by one-way ANOVA (P < 0.05).

4. Conclusions

All tested concentrations of D-Ac-106 (up to 100 μ M) and D-106 at 50 or 100 μ M effectively stimulated the radicle development and growth. D-tryptophan-derived urease inhibitors are promising molecules for use in urea-based formulations, since in addition to inhibiting soil ureases, the compounds have a beneficial effect on root development. The millet germination process, nor the antioxidant metabolism enzymes, were not negatively affected by the derivatives, nor by NBPT. These data are an indication that new urease inhibitors derived from D-tryptophan do not affect plant metabolism at the tested concentrations. However, further studies are needed to confirm the safe use of these compounds.

References

- Beretta-Blanco, A., Pérez, O., & Carrasco-Letelier, L. (2019). Soil quality decrease over 13 years of agricultural production. *Nutrient Cycling in Agroecosystems*, 114(1), 45-55.
- Bewley, J.D., Black, M., 1994. *Seeds: Physiology of Development and Germination*. Plenum Press, New York. 430P.
- Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 72(1-2), 248-254.
- Carvalho, D.B de; Carvalho, R.I.N de. Qualidade fisiológica de sementes de guanxuma em influência do envelhecimento acelerado e da luz. *Acta Scientiarum. Agronomy*. 2009; 31 (3): 489-494.
- Desta, B., & Amare, G. (2021). Paclobutrazol as a plant growth regulator. *Chemical and Biological Technologies in Agriculture*, 8(1), 1-15.
- Dominchin, M. F., Verdenelli, R. A., Berger, M. G., Aoki, A., & Meriles, J. M. (2021). Impact of N-fertilization and peanut shell biochar on soil microbial community structure and enzyme activities in a Typic Haplustoll under different management practices. *European Journal of Soil Biology*, 104, 103298.
- Giannopolitis, C. N., & Ries, S. K., 1977. Superoxide dismutases: I. Occurrence in higher plants. *Plant Physiology*. 59(2), 309-314.
- Grant, W. F., 1982. Chromosome aberration assays in *Allium*. A report of the U.S. environmental protection agency. Gene-Tox program. *Mutation Research*, 99, 273- 291.
- Grant, W. F., 1994. The present status of higher plant bioassays for detection of environmental mutagens. *Mutat. Res.* 310, 175–185.
- Horta, L. P., Braga, M. R., Lemos-Filho, J. P., & Modolo, L. V., 2014. Organ-coordinated response of early post-germination mahogany seedlings to drought. *Tree Physiology*. 34(4), 355-366.
- Kolukisaoglu, Ü., & Suarez, J. (2017). D-Amino acids in plants: new insights and aspects, but also more open questions. *Amino Acid-New Insights and Roles in Plant and Animal*, 10.
- Labouriau, L.G., 1983. *A Germinação das Sementes*. Washington D.C, Secretaria Geral da Organização dos Estados Americanos.

- Lambais, M. R., CURY, J. de C., Maluche-Baretta, C. R., Bull, R. de C., 2005. Diversidade microbiana nos solos: definindo novos paradigmas. *Tópicos em Ciências do Solo*. 4, 42-84.
- Lemos Filho, J.P., Guerra, S.T.M., Lovato, M.B., Scotti, M.R.M.M.L., 1997. Germinação de sementes de *Senna macranthera*, *Senna multijuga* e *Stryphnodendron polyphyllum*. *Pesquisa Agropecuária Brasileira*. 32(4), 357-361.
- Luo, Y., Liang, J., Zeng, G., Chen, M., Mo, D., Li, G., & Zhang, D. (2018). Seed germination test for toxicity evaluation of compost: Its roles, problems and prospects. *Waste Management*, 71, 109-114.
- Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Science*. 7, 405-410.
- Parween, T., Jan, S., Mahmooduzzafar, S., Fatma, T., & Siddiqui, Z. H. (2016). Selective effect of pesticides on plant—A review. *Critical reviews in food science and nutrition*, 56(1), 160-179.
- Rathore, S., & Singh, K. (2017). A critical optimization study on hydrothermal treatment for decortication of pearl millet to improve its consumption efficiency. *Journal of Food Measurement and Characterization*, 11(3), 1501-1515.
- Rosenthal, G. A. (1991). The biochemical basis for the deleterious effects of L-canavanine. *Phytochemistry*, 30(4), 1055-1058.
- Soltani E; Ghaderi-Far F; Baskin CC; Baskin JM. Problems with using mean germination time to calculate rate of seed germination. *Aust J Bot*. 2015; 63: 631-635.
- Zhang, G., & Sun, H. J. (2014). Racemization in reverse: evidence that D-amino acid toxicity on Earth is controlled by bacteria with racemases. *Plos one*, 9(3), e92101.
- Zhang, R., Zhang, H., Tu, C., Hu, X., Li, L., Luo, Y., & Christie, P. (2015). Phytotoxicity of ZnO nanoparticles and the released Zn (II) ion to corn (*Zea mays* L.) and cucumber (*Cucumis sativus* L.) during germination. *Environmental Science and Pollution Research*, 22(14), 11109-11117.

Chapter 3

Influence of urease inhibitors on the early stages of millet development and their contribution for the production of root exudates that modulate nitrification *in vitro*

Abstract

Plants exert great influences on the global nitrogen cycle by releasing exudates from roots, which are capable of inhibiting or stimulating nitrification and denitrification. Nitrification inhibitors are of great interest as they contribute to an increase of the nitrogen use and efficiency (NUE) in the soil, a decrease of ammonium oxidation to form nitrate, ultimately mitigating the nitrogen loss due to nitrate leaching. Likewise, urease inhibitors are substances used in agriculture to improve NUE when urea is used as a fertilizer. NBPT is an extensively used urease inhibitor and it is known to affect plant metabolism. Thus, the aim of this work was to determine the effect of pearl millet root exudates hydroponically generated in the presence of urea plus new urease inhibitors derived from amino acid (D-106 and D-Ac-106) or NBPT on the nitrification potential in latossolo vermelho distrófico soil. The root exudates resulting from NBPT or amino acid derivatives treatments decreased the nitrification potential of the soil in comparison with urea treatment devoid of urease inhibitors. NBPT and D-106 treatment decreased the oxidation of ammonium by 37.8 and 43.5% respectively. D-Ac-106 treatment showed outstanding results of 86.9% nitrification inhibition. The use of urease and nitrification inhibitors in urea formulations is known to benefit crop productions. More studies are needed to identify the mechanism of action of these substances and, thus, allow their safe use for the environment.

Keywords: Nitrification inhibitors, Urease inhibitors, NUE, Root exudates, Pearl millet.

1. Introduction

Urea is the most used nitrogen fertilizer worldwide (Wang et al., 2020). However, when applied to the soil, urea is hydrolyzed into carbamate and ammonia by extracellular ureases present in the soil, causing great economic losses, besides contributing to environmental pollution (Soares et al., 2012). NBPT is a commercial urease inhibitor used in agriculture to slow down urea hydrolysis in the soil and thus avoid large N losses via ammonia volatilization. Thus, the search for new urease inhibitors have increased in recent years (Camargo et al., 2022). Some studies have evaluated the safety of NBPT (Ray et al., 2021), as well as its effects on plants and animals. However, very little is known about promising new urease inhibitors.

Some works have shown the action of NBPT on plant metabolism (Artola et al., 2011; Cruchaga et al., 2011). Besides inhibiting ureases in plants (Cruchaga et al., 2013), NBPT downregulated shikimate pathway transcripts and upregulated the phenylpropanoid pathway, which is responsible for the biosynthesis of secondary metabolites that play key roles in the plant defense against (a)biotic stresses, (Zanin et al., 2016).

Changes in plant metabolism, promoted by xenobiotics, such as urease inhibitors, are eligible to affect the composition of root exudates, known to play important roles in the plant communication with the soil microbiota (Pii et al., 2015). Qualitative and quantitative changes in exudates constitution may shape specific groups of microorganisms. This is because the great diversity of taxa in the rhizosphere includes a relatively high proportion of strains adapted to specialized metabolic niches (Zhalnina et al., 2018).

Understanding the extent of how xenobiotics affect plants and consequently influence the microbiota is important, given the many benefits existing in the soil-plant relationship. Plants can benefit from the rhizospheric microbiota as it may boost the plant defense against pathogens and nutrient acquisition as well (Yadav et al., 2021). Indeed, the acquisition of nutrients by plants is influenced by biogeochemical cycles driven by the soil microbiota.

Nitrogen metabolism is strongly influenced by root exudates and soil microbiota (Coskun et al., 2017). Nitrification is a key step in the soil nitrogen cycle and widely studied in both natural and agricultural systems (Beeckman et al., 2018; Scarlet et al., 2021). The autotrophic nitrification process involves the oxidation of ammonium (NH_4^+)

to nitrite (NO_2^-) and is driven by ammonium oxidizing bacteria (AOB) and archaea (AOA) (Porsser et al., 2008). The nitrite formed is further oxidized to nitrate (NO_3^-) by nitrifying bacteria, which results in a net loss of N by nitrate leaching and/or denitrification process that releases N to the atmosphere (as N_2 and/or N_2O). NBPT acts on the prokaryotic community and is also able to modulate functional groups (Li et al., 2019; Liu et al., 2022). For instance, NBPT was shown to decrease the abundance of AOB groups and increase the abundance of AOA ones (Fan et al., 2018).

Understanding how xenobiotics modify plant metabolism and influence nutrient cycles and processes such as nitrification is an important step towards achieving agricultural sustainability. Thus, this work aimed to investigate the effect of new urease inhibitors on the initial development of Pearl millet cultivated under different nitrogen sources and investigated the effect of exudates from pearl millet roots hydroponically cultivated with urea and new urease inhibitors (D-106 and D-Ac-106) or NBPT, on the nitrification potential of the soil and plant development as well .

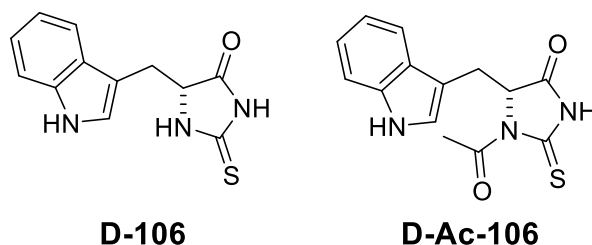


Figure 1. Structures of acetyl (thio)hydantoin and thiohydantoin.

2. Materials and methods

2.1 Growth conditions of pearl millet

Millet seeds (BRS 1501) were pre-germinated for 24 h in a 0.5% Nystatin® solution at 25°C and 12 h photoperiod. The pre-germinated seeds were wrapped in filter paper and kept for 48 h in the same chamber under the same conditions. Uniform-sized seedlings were then placed in a nutrient solution containing (µM): KCl 10; CaSO₄ 1000; MgSO₄ 200; KH₂PO₄ 350; NaFe-EDTA 40; H₃BO₃ 5; MnSO₄ 0.4; ZnSO₄ 0.4; CuSO₄ 0.1; Na₂MoO 0.1 in pots of 300 mL with 5 plants each. Nitrogen was added in the hydroponic medium either in the form of 1 mM urea CO(NH₂)₂ (urea treatment) or as 1 mM nitrate Ca(NO₃)₂ (nitrate treatment) and the pH of solution was adjusted to pH 6.0. Plants were also exposed to N-free solution. The urease inhibitors NBPT, D-106 and D-Ac-106 (at 1 µM) were prepared in ethanol containing DMSO and PEG-400 and was applied to nutrient solution. Thus, the treatments were: urease inhibitors (present or not) in the nutrient solution of: urea treatment, nitrate treatment and N-free treatment. The material was collected from 5-day-old pearl millet plants exposed for 8 days to the different treatments for morphological evaluation and chloroplast pigments or stored in liquid nitrogen for further analyses.

2.2 Chloroplast pigments and chlorophyll fluorescence

To quantify the chloroplast pigments, 0.1 g of fresh leaves were immersed in 5 mL of DMSO and kept for 48 h in the dark. The leaf homogenates were monitored at 649 and 665 nm to determine the levels of chlorophylls *a* and *b*, respectively. Measurements at 470 nm were used to calculate the carotenoids levels, according to Lichtenthaler (1987). The chlorophyll fluorescence was measured in fully expanded leaves of seedlings under 8 days of treatment using a Modulated Fluorometer (MINI PAM, Walz Mess und Regeltechnik). Millet seedlings were dark-adapted for 30 min prior to determining the maximum quantum yield of photosystem II (Fv/Fm). The maximum apparent electron transport rate (ETR_{max}, µmol electrons m⁻² s⁻¹) was calculated from light curves (Rascher et al., 2000).

2.3 Urea quantification

For determination of urea content, 100 mg of frozen leaves or roots were boiled and frozen for urease inactivation (Witte et al., 2002). Subsequently, 20 μL of the aqueous extract were added to a colorimetric reagent (Kyllingsbaek 1975) consisting of diacetyl monoxime (248 mM), thiosemicarbazide, (27.4 mM), 51% H_3PO_4 and 2% H_2SO_4 . The system was incubated at 100 $^{\circ}\text{C}$ for 30 min, then cooled down to room temperature for 15 min. Samples were monitored at 527 nm to quantify urea from a standard curve.

2.4 Quantification of lipid hydroperoxide

The quantification of the lipid hydroperoxide (H_2O_2) was performed according to Delong et al. (2002). Plant material (0.3 g) was ground in the presence of PVPP and resuspended in 0.01% (w/v) butylated hydroxytoluene (BHT) prepared in 80% ethanol. The homogenates were centrifuged and an aliquot (25 μL) was taken and mixed with 25 μL of methanol, or 25 μL methanol plus 10 mM triphenylphosphine for the control reaction. A freshly prepared FOX reagent (100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 90% methanol, 4 mM butylated hydroxytoluene, and 110 mM perchloric acid) was added to each reaction containing or not plant extract and the mixture was incubated at room temperature for 30 min followed by measurements of absorbance at 560 nm. The lipid hydroperoxide content was expressed as nM of H_2O_2 per mg of fresh mass, based on a standard curve of hydrogen peroxide.

2.5 Effect of exudates from millet roots on the potential of soil nitrification

The nutrient solution was harvested at the 8th day of millet treatment (corresponding to the collection of 48 hours of exudates). Because the nutrient solution was changed every other day, all compounds exuded from the millet roots at the 8th resulted from a 48h treatment. Each root exudate was vacuum filtered and lyophilized. The resulting powder was resuspended in 80% methanol, lyophilized again and resuspended in DMSO to obtain a solution at 100 mg/mL. The nitrification test was performed according to Janke et al (2018), with modifications. Latossolo vermelho distrófico soil (2.5 g) was activated for 24 h in the dark at 60% of field capacity, together with 50 μL of the exudate resuspended in 950 μL of ultrapure water. As a negative control, 50 μL DMSO was added to 950 μL ultrapure water. Then, 20

mL of 3.8 mM ammonium sulfate solution prepared in 1 mM phosphate buffer (pH 7.2) was added to the soil, plus an extra 50 μ L of diluted exudate. The system was incubated in the dark for 24 h at 180 rpm on an orbital shaker. One aliquot (1 mL) of each system was collected and centrifuged at 3000 rpm for 10 min. The nitrite formed was quantified by the sulfanilamide method according to Griess et al. (1879).

2.6 Statistical analysis

Data were evaluated for normality and homoscedasticity and analyzed by ANOVA. In the initial development, the urea and lipid hydroperoxide content were used after the Dunnett's test and the treatments were compared with the control (Inhibitor-free) in the GraphPAD prism 7.0 software. The Scott Knott test was performed in the R 4.2.1 program for the remaining data.

3. Results

3.1 Millet early development and chloroplast pigments levels

Millet seedlings grown in a nutrient solution devoid of N showed poor shoot development and greater root growth. Urea or nitrate promoted shoot growth, 106 and 102 % respectively, as well as increased pigment production (Table 1). The treatment containing NBPT + urea resulted in shorter shoot and lower root dry mass (Figure 3). There was no difference between treatment with nitrate and nitrate + NBPT. (Thio)hydantoins, as well as NBPT, did not interfere with any parameters of seedlings growing in the absence of nitrogen. However, they influenced both shoot and root development in plants grown with urea or nitrate (Figure 3). Thus, D-Ac-106 reduced shoot length and fresh mass, length, fresh and dry mass of roots, when treated with urea, and root length, when cultivated with nitrate. (Thio)hydantoin D, on the other hand, did not show any effect on the aerial part, under cultivation with nitrate or urea, but it reduced the length and fresh mass of roots treated with urea (Figure 3). While D-Ac-106 interfered with the production of chloroplast pigments in plants grown with nitrate, there was no significant difference in seedlings treated with (thio)hydantoin D (table 1).

Only NBPT was able to reduce the maximum photosynthetic potential (F_v/F_m) in pearl millet under urea culture (Figure 3A). Furthermore, NBPT and D-Ac-106 reduced the maximum electron transport (ETR_{max}) when cultured with urea (Figure 4B). Under cultivation with nitrate, despite D-Ac-106 presenting the lowest average, there was no significant difference in relation to the control.

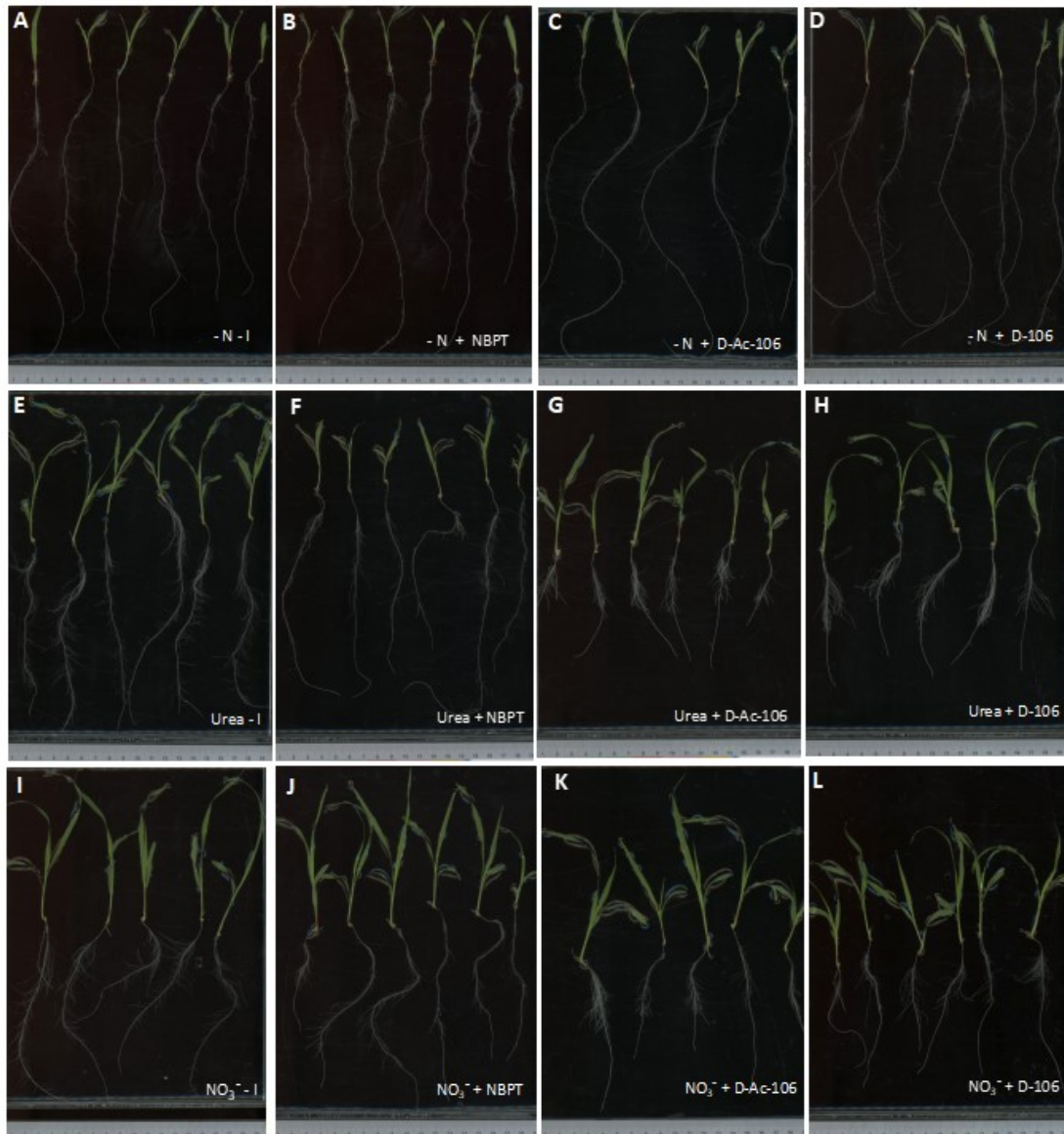


Figure 2. Pearl millet cultured for 8 days in nutrient solution without nitrogen (NF) (**A-D**) or in different sources of N at 1mM: urea (U) (**E-H**) or nitrate (NO_3^-) (**I-L**) in the absence or presence of inhibitors at 1 μM .

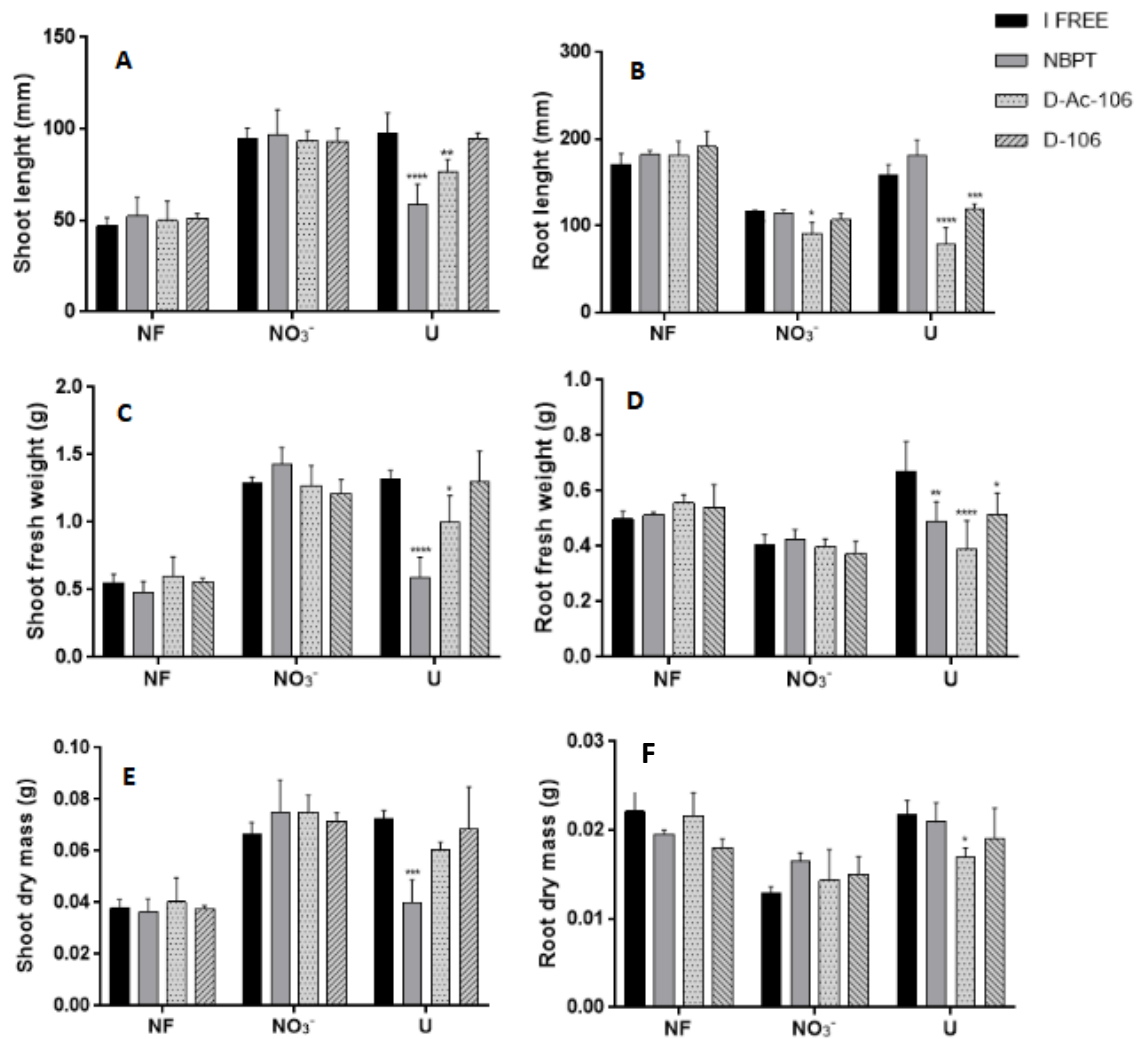


Figure 3. Pearl millet shoot length (A), root length (B), shoot fresh weight (C), root fresh weight (D), shoot dry mass (E) and root dry mass (F) cultured for 8 days in nutrient solution without nitrogen (NF) or in different sources of N at 1mM: nitrate (NO_3^-) or urea (U) in the absence or presence of inhibitors at 1 μM . Bars are mean $\pm$ standard deviation, n=3. Asterisks represent statistical difference in relation to control (IF) by Dunnett's test at 0.05 of significance.

Table 1. Chloroplast pigments (mg/mg) in pearl millet leaves cultivated for 8 days in different nitrogen sources with or without urease inhibitors.

Tratament	Chlorophyll a	Chlorophyll b	Carotenoids	Chlorophyll (a/b)	Chlorophyll/Carotenoids
NF	2,70 ± 0,1 c	1,23 ± 0,1 b	0,72 ± 0,0 b	2,20 ± 0,2 b	0,19 ± 0,0 a
NF + NBPT	2,57 ± 0,0 c	1,26 ± 0,0 b	0,67 ± 0,0 b	2,04 ± 0,0 b	0,18 ± 0,0 a
NF + D-Ac-106	3,21 ± 0,2 b	1,39 ± 0,1 b	0,78 ± 0,0 b	2,30 ± 0,1 b	0,17 ± 0,0 a
NF + D-106	2,76 ± 0,0 c	1,17 ± 0,1 b	0,76 ± 0,0 b	2,37 ± 0,2 b	0,19 ± 0,0 a
Ureia	3,81 ± 0,3 a	1,56 ± 0,1 a	0,91 ± 0,1 a	2,45 ± 0,1 a	0,17 ± 0,0 a
Urea + NBPT	2,56 ± 0,3 c	1,27 ± 0,1 b	0,66 ± 0,1 b	2,02 ± 0,1 b	0,17 ± 0,0 a
Urea + D-Ac-106	3,79 ± 0,4 a	1,55 ± 0,1 a	0,91 ± 0,2 a	2,44 ± 0,3 a	0,17 ± 0,0 a
Urea + D-106	3,83 ± 0,3 a	1,49 ± 0,1 a	0,96 ± 0,1 a	2,56 ± 0,0 a	0,18 ± 0,0 a
Nitrate	3,81 ± 0,4 a	1,49 ± 0,1 a	0,97 ± 0,1 a	2,55 ± 0,1 a	0,18 ± 0,0 a
Nitrate + NBPT	3,66 ± 0,7 a	1,40 ± 0,2 a	0,92 ± 0,2 a	2,54 ± 0,2 a	0,18 ± 0,0 a
Nitrate + D-Ac-106	3,29 ± 0,7 b	1,30 ± 0,2 b	0,84 ± 0,1 b	2,52 ± 0,1 a	0,19 ± 0,0 a
Nitrate + D-106	3,57 ± 0,3 a	1,36 ± 0,1 b	0,93 ± 0,1 a	2,63 ± 0,1 a	0,19 ± 0,0 a

Mean ± standard deviation (n=3). Letters represent statistical differences by the ScottKnott test at 0.05 significance.

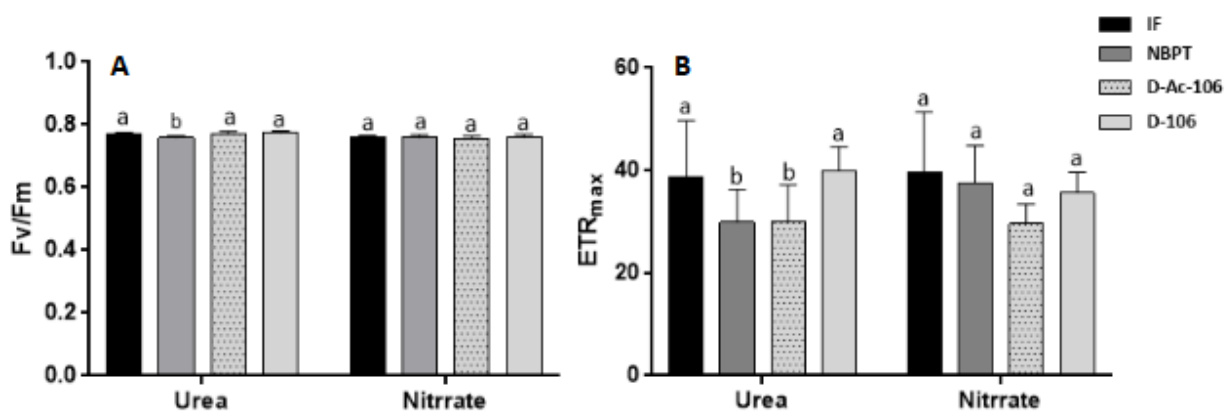


Figure 4. Chlorophyll fluorescence parameters of **A**- Photochemical efficiency (Fv/Fm) of Pearl millet leaves, **B** - maximum apparent electron transport rate (ETR_{max}) ($\mu\text{M e}^- \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Values are indicated as mean ± standard deviation, n = 6. Different letters represent the results are statistically significant at $P \leq 0.05$ by Scott-Knott test.

3.2 Urea content

NBPT promoted a large accumulation of urea in shoot (Figure 5), the same did not occur in the treatment with (thio)hydantoin. There was no difference between treatment with inhibitors and treatment with only urea in the roots.

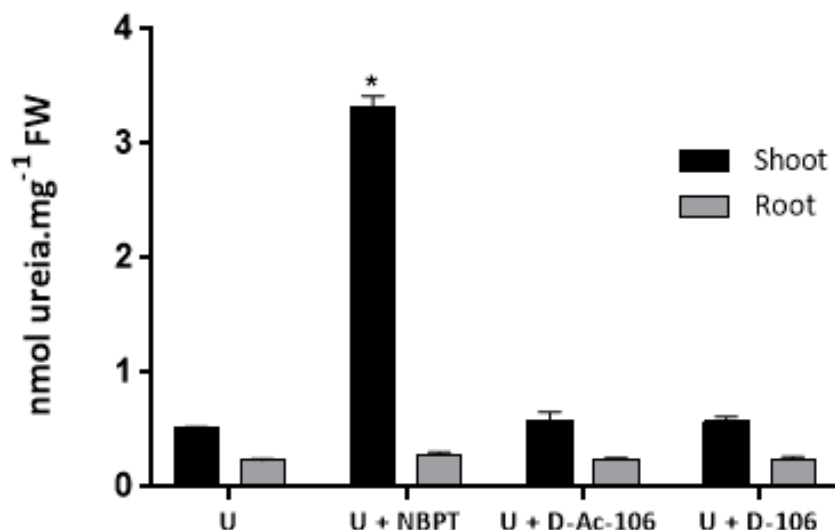


Figure 5. Urea content in shoots and roots of seedlings grown with urea in the absence or presence of urease inhibitors for 8 days. Bars represent mean $\pm$ standard deviation, $n=3$. Asterisk represents a significant difference ($p \leq 0.05$) compared by dunnett's test between treatment without inhibitor.

3.3 Lipid hydroperoxide and soil nitrification potential

Higher lipid hydroperoxide content was evidenced in the roots than the shoot. However, the treatment with NBPT or (thio)hydantoin derived from amino acids did not further stimulate the accumulation of lipid hydroperoxide in plants (Figure 6). Exudates from seedlings treated with any of the three urease inhibitors significantly decreased nitrite production from ammonium in the soil, therefore inhibiting the nitrification process (Figure 7). The greatest inhibition of nitrification ($> 80\%$) was achieved with the use of exudates from plants treated with D-Ac-106, in relation to the control.

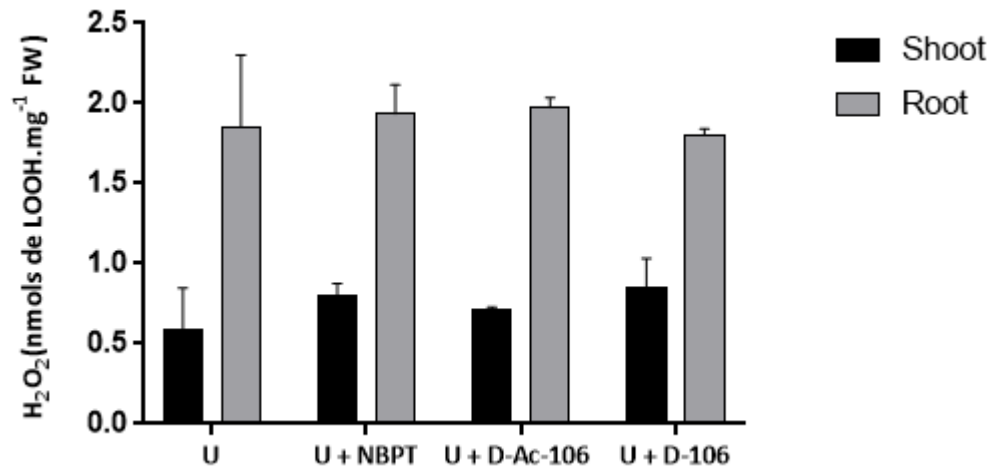


Figure 6. Lipid hydroperoxide content in shoots and roots of seedlings grown with urea in the absence or presence of urease inhibitors for 8 days. Bars represent mean $\pm$ SD, $n=3$. Asterisk represents a significant difference ($p \leq 0.05$) compared to treatment without inhibitor (IF).

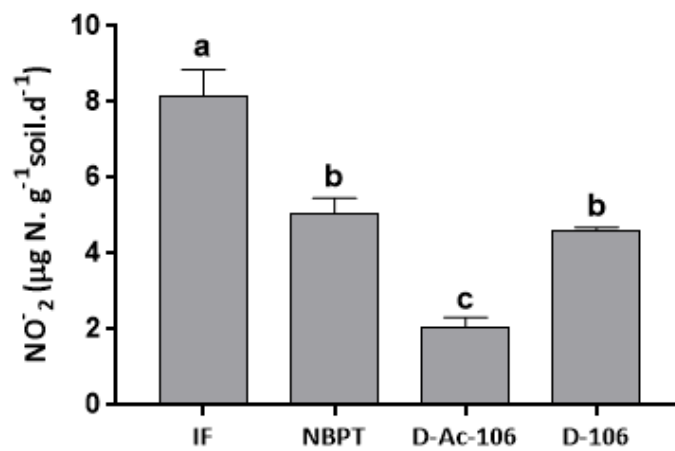


Figure 7. Soil nitrite content after treatment for 48 hours with exudates from plants cultivated with urea in the absence (IF) or presence of urease inhibitors. Bars represent mean $\pm$ SD, $n=3$. Different letters represent a significant difference ($P \leq 0.05$) by the ScottKnott test.

4. Discussion

The action of the NBPT inhibitor together with urea has a great influence on plant metabolism. The millet growth arrest and accumulation of urea in plant leaves upon association of urea with NBPT have been reported elsewhere within 24 h post-treatment (Zanin, et al., 2015; Zanin et al., 2016). The accumulation of urea is possibly due to inhibition of plant ureases by NBPT, since it was reported the ability of plant roots to uptake this urease inhibitor (Cruchaga et al., 2011). Ureases are the only enzymes capable of converting urea to ammonium and carbamate at a very high rate (Frankenberger and Tabatabai, 1982). The lack of urease activity in plant cells therefore leads to the accumulation of urea in the cytosol of shoot and root cells and cell walls and membranes (Aguetoni et al., 2009). NBPT appears to have great inhibition of shoot ureases, or during shoot germination. Thus, the poor development of millet seedlings treated with urea+NBPT may be due to the inability of plant cells to further convert the organic N present in urease into an inorganic form (NH_4^+) for its assimilation. L-arginine is the precursor for endogenous formation of urea and one of the main nitrogen reservoirs in seeds (Zonia et al., 1995). The activity of arginase increases during the germination, which leads to an increment in urease content. Thus, the urease activity is extremely important for endogenous N cycling as well.

Despite the lowering of photosynthetic rates in plants upon the treatment with NBPT plus nitrate (Crunchaga et al., 2013), no changes were observed in shoot and root development in seedlings grown with this N source in the presence of NBPT. The presence of (thio)hydantoins derived from aminoacids in the hydroponic solution did not affect the millet urea content in roots and shoots in comparison to plants grown in the presence of urea devoid of (thio)hydantoins (Figure 5). Considering the proven ability of such (thio)hydantoins to inhibit urease from plants in *in vitro* experiments, one can suppose that such urease inhibitors were not uptaken by millet roots. Instead, both amino acid derivatives promoted major changes in the architecture of seedlings grown in a solution containing urea and nitrate (Figure 3). The formation of lateral roots in millet was considerably stimulated by D-106 and D-Ac-106 (Figure 2).

Root exudates from plants treated with urea devoid of any urease inhibitor significantly increased the nitrification potential of the soil tested. This is possibly due to the increased metabolism of the microbiota, which uses the numerous compounds present in the exudates as an energy source. Exudates from plants treated with any of

the urease inhibitors significantly reduced the soil nitrification potential (Figure 6). The contrasting results obtained for NBPT and (thio)hydantoins may be indicative of differences in the chemical constitution of exudates due to the presence of distinct classes of urease inhibitors. This hypothesis must be tested by further evaluating the chemical constitution of exudates using proper analytical equipment. The NBPT likely prevented the millet development by preventing the seedlings from using the main nitrogen source provided (urea). Thus, low production of chlorophylls (N-containing substance) was noted upon NBPT treatment (Table 1). Nitrification is one of the main processes related to the emission of nitrous oxide (N_2O) (Well et al., 2005). The rhizosphere is the main site of N_2O emission, mainly due to the great activity of the microbiota in this region (Xing et al., 2021), which receives a constant source of carbon and has a rapid consumption of oxygen (Qial et al., 2014). The conversion of ammonium to nitrite in the soil is greatly influenced by the composition of the exudates, since plants have the ability to release nitrification inhibitors (Sun et al., 2016). Plants under stress also increase the exudation of substances from roots, such as phenolics, with high capacity to inhibit nitrification (Ghatak et al., 2022). The present work evidences that the treatment with (thio)hydantoins did not cause plant stress as the content of lipid hydroperoxides did not increase (Figure 5). The increment in the levels of lipid peroxides is an effective way to determine whether the plants are under stress (de Azevedo Neto et al., 2006; Zhao et al., 2012; Killi et al., 2020). However, a very stress-sensitive photosynthetic parameter, such as chlorophyll fluorescence, was affected by acetylated (thio)hydantoins (Figure 3). Photosystem II is very sensitive to stress, so it is an important parameter used in the assessment of stress, such as those caused by contaminants (Lee et al., 2021; Ogo et al., 2022). Only NBPT in plants cultivated with urea was able to reduce the potential quantum yield (F_v/F_m). This possibly occurred due to the decrease of chloroplast pigments (Xu et al., 2010).

Despite the reduction promoted by NBPT and D-Ac-106 in ETR_{max} in the urea treatment, both compounds did not affect this photosynthetic parameter under nitrate nutrition (Figure 4). One possibility is that D-106 and D-Ac-106 may act as signaling molecules, stimulating the release of nitrification-inhibiting compounds from millet roots. For this, further studies should be carried out to confirm or refute this hypothesis.

5. Conclusion

The worldwide used urease inhibitor NBPT negatively affected some millet traits, such as pigment production under urea nutrition. As expected, the urease inhibitors did not impair millet traits upon plant cultivation with nitrate as the N source. (Thio)hydantoins improved the millet development by inducing the formation of lateral roots, an architecture change that may benefit plants concerning the uptake of water and nutrients from soil. The acetylated (thio)hydantoin were more effective suggesting that the acetyl group is pivotal for such activity. Root exudates from plants treated with all inhibitors reduced the soil nitrification potential. Exudates promote an effect on the rhizosphere, which is the main site of nitrous oxide emission, thus, a key site for future studies to promote a more sustainable agriculture.

References

- Aguetoni Cambuí, C., Gaspar, M. L., & Mercier, H. (2009). Detection of urease in the cell wall and membranes from leaf tissues of bromeliad species. *Physiologia plantarum*, 136(1), 86-93.
- Artola, E., Cruchaga, S., Ariz, I., Moran, J. F., Garnica, M., Houdusse, F., ... & Aparicio-Tejo, P. M. (2011). Effect of N-(n-butyl) thiophosphoric triamide on urea metabolism and the assimilation of ammonium by *Triticum aestivum* L. *Plant growth regulation*, 63(1), 73-79.
- Beeckman, F., Motte, H., & Beeckman, T. (2018). Nitrification in agricultural soils: impact, actors and mitigation. *Current opinion in Biotechnology*, 50, 166-173.
- Camargo, P. G., Fabris, M., Nakamae, M. Y. T., de Freitas Oliveira, B. G., da Silva Lima, C. H., de Fátima, Â., ... & Macedo Jr, F. (2022). (Thio)hydantoins and hydantoins derived from amino acids as potent urease inhibitors: Inhibitory activity and ligand-target interactions. *Chemico-Biological Interactions*, 365, 110045.
- Coskun, D., Britto, D. T., Shi, W., & Kronzucker, H. J. (2017). How plant root exudates shape the nitrogen cycle. *Trends in Plant Science*, 22(8), 661-673.
- Cruchaga, S., Lasá, B., Jauregui, I., González-Murua, C., Aparicio-Tejo, P. M., & Ariz, I. (2013). Inhibition of endogenous urease activity by NBPT application reveals differential N metabolism responses to ammonium or nitrate nutrition in pea plants: a physiological study. *Plant and soil*, 373(1), 813-827.
- Cruchaga, S., Artola, E., Lasá, B., Ariz, I., Irigoyen, I., Moran, J. F., & Aparicio-Tejo, P. M. (2011). Short term physiological implications of NBPT application on the N metabolism of *Pisum sativum* and *Spinacea oleracea*. *Journal of plant physiology*, 168(4), 329-336.
- de Azevedo Neto, A. D., Prisco, J. T., Enéas-Filho, J., de Abreu, C. E. B., & Gomes-Filho, E. (2006). Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environmental and Experimental Botany*, 56(1), 87-94.
- DeLong, J. M., Prange, R. K., Hodges, D. M., Forney, C. F., Bishop, M. C., & Quilliam, M. (2002). Using a modified ferrous oxidation– xylene orange (FOX) assay for detection of lipid hydroperoxides in plant tissue. *Journal of Agricultural and Food Chemistry*, 50(2), 248-254.
- Fan, X., Yin, C., Yan, G., Cui, P., Shen, Q., Wang, Q., ... & Liang, Y. (2018). The

- contrasting effects of N-(n-butyl) thiophosphoric triamide (NBPT) on N₂O emissions in arable soils differing in pH are underlain by complex microbial mechanisms. *Science of the total environment*, 642, 155-167.
- Frankenberger, W. T., & Tabatabai, M. A. (1982). Amidase and urease activities in plants. *Plant and Soil*, 64(2), 153-166.
- Janke, C. K., Wendling, L. A., & Fujinuma, R. (2018). Biological nitrification inhibition by root exudates of native species, *Hibiscus splendens* and *Solanum echinatum*. *PeerJ*, 6, e4960.
- Lee, H., Depuydt, S., Shin, K., Choi, S., Kim, G., Lee, Y. H., ... & Park, J. (2021). Assessment of Various Toxicity Endpoints in Duckweed (*Lemna minor*) at the Physiological, Biochemical, and Molecular Levels as a Measure of Diuron Stress. *Biology*, 10(7), 684.
- Ghatak, A., Schindler, F., Bachmann, G., Engelmeier, D., Bajaj, P., Brenner, M., ... & Weckwerth, W. (2022). Root exudation of contrasting drought-stressed pearl millet genotypes conveys varying biological nitrification inhibition (BNI) activity. *Biology and fertility of soils*, 58(3), 291-306.
- Griess P. Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt "Ueber einige Azoverbindungen". *Ber Dtsch Chem Ges.* 1879;12:426–8.
- Killi, D., Raschi, A., & Bussotti, F. (2020). Lipid peroxidation and chlorophyll fluorescence of photosystem II performance during drought and heat stress is associated with the antioxidant capacities of C3 sunflower and C4 maize varieties. *International journal of molecular sciences*, 21(14), 4846.
- Li, W., Xiao, Q., Hu, C., Liu, B., & Sun, R. (2019). A comparison of the efficiency of different urease inhibitors and their effects on soil prokaryotic community in a short-term incubation experiment. *Geoderma*, 354, 113877.
- Liu, C., Zhang, Y., Liu, H., Liu, X., Ren, D., Wang, L., ... & Zhang, M. (2022). Fertilizer stabilizers reduce nitrous oxide emissions from agricultural soil by targeting microbial nitrogen transformations. *Science of The Total Environment*, 806, 151225.
- Ogo, H. A., Tang, N., Li, X., Gao, X., & Xing, W. (2022). Combined toxicity of microplastic and lead on submerged macrophytes. *Chemosphere*, 295, 133956.
- Pii et al., , Y. Pii, T. Mimmo, N. Tomasi, R. Terzano, S. Cesco, C. Crecchio (2015) Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting Rhizobacteria on nutrient acquisition process. A review. *Biol. Fertil.*

- Soils, 51, pp. 403-415.
- Qiao, Y., Miao, S., Han, X., You, M., Zhu, X., & Horwath, W. R. (2014). The effect of fertilizer practices on N balance and global warming potential of maize–soybean–wheat rotations in Northeastern China. *Field Crops Research*, 161, 98-106.
- Rascher, U., Liebig, M., & Lüttge, U. (2000). Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. *Plant, Cell & Environment*, 23(12), 1397-1405.
- Ray, A., Nkwonta, C., Forrestal, P., Danaher, M., Richards, K., O'Callaghan, T., ... & Cummins, E. (2021). Current knowledge on urease and nitrification inhibitors technology and their safety. *Reviews on Environmental Health*, 36(4), 477-491.
- Scarlett, K., Denman, S., Clark, D. R., Forster, J., Vanguelova, E., Brown, N., & Whitby, C. (2021). Relationships between nitrogen cycling microbial community abundance and composition reveal the indirect effect of soil pH on oak decline. *The ISME journal*, 15(3), 623-635.
- Soares, J. R., Cantarella, H., & de Campos Menegale, M. L. (2012). Ammonia volatilization losses from surface-applied urea with urease and nitrification inhibitors. *Soil biology and biochemistry*, 52, 82-89.
- Sun, L., Lu, Y., Yu, F., Kronzucker, H. J., & Shi, W. (2016). Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. *New Phytologist*, 212(3), 646-656.
- Wang, H., Köbke, S., & Dittert, K. (2020). Use of urease and nitrification inhibitors to reduce gaseous nitrogen emissions from fertilizers containing ammonium nitrate and urea. *Global Ecology and Conservation*, 22, e00933.
- Zanin, L., Venuti, S., Tomasi, N., Zamboni, A., De Brito Francisco, R. M., Varanini, Z., & Pinton, R. (2016). Short-term treatment with the urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT) alters urea assimilation and modulates transcriptional profiles of genes involved in primary and secondary metabolism in maize seedlings. *Frontiers in plant science*, 7, 845.
- Zhalnina, K., Louie, K. B., Hao, Z., Mansoori, N., da Rocha, U. N., Shi, S., ... & Brodie, E. L. (2018). Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nature microbiology*, 3(4), 470-480.
- Zonia, L. E., Stebbins, N. E., and Polacco, J. C. (1995). Essential role of urease in germination of nitrogen-limited *Arabidopsis thaliana* seeds. *Plant Physiol.* 107,

1097–1103.

- Kyllingsbæk, A. (1975). Extraction and colorimetric determination of urea in plants. *Acta Agriculturae Scandinavica*, 25(2), 109-112.
- Well, R., Flessa, H., Jaradat, F., Toyoda, S., & Yoshida, N. (2005). Measurement of isotopomer signatures of N₂O in groundwater. *Journal of Geophysical Research: Biogeosciences*, 110(G2).
- Witte, C. P., Tiller, S. A., Taylor, M. A., & Davies, H. V. (2002). Leaf urea metabolism in potato. Urease activity profile and patterns of recovery and distribution of ¹⁵N after foliar urea application in wild-type and urease-antisense transgenics. *Plant Physiology*, 128(3), 1129-1136.
- Xing, L., Qin, W., Manevski, K., Zhang, Y., Hu, C., Zhang, L., ... & Qin, S. (2021). An improved microelectrode method reveals significant emission of nitrous oxide from the rhizosphere of a long-term fertilized soil in the North China Plain. *Science of the Total Environment*, 783, 147011.
- Xu, Q. S., Hu, J. Z., Xie, K. B., Yang, H. Y., Du, K. H., & Shi, G. X. (2010). Accumulation and acute toxicity of silver in *Potamogeton crispus* L. *Journal of hazardous materials*, 173(1-3), 186-193.
- Yadav, A. N., Kour, D., Kaur, T., Devi, R., Yadav, A., Dikilitas, M., ... & Saxena, A. K. (2021). Biodiversity, and biotechnological contribution of beneficial soil microbiomes for nutrient cycling, plant growth improvement and nutrient uptake. *Biocatalysis and Agricultural Biotechnology*, 33, 102009.
- Zhao, L., Peng, B., Hernandez-Viezcas, J. A., Rico, C., Sun, Y., Peralta-Videa, J. R., ... & Gardea-Torresdey, J. L. (2012). Stress response and tolerance of *Zea mays* to CeO₂ nanoparticles: cross talk among H₂O₂, heat shock protein, and lipid peroxidation. *ACS nano*, 6(11), 9615-9622.

Chapter 4

**Amino acid-derived urease inhibitors
increase pearl millet nutrient uptake by
changing the composition and
structure of the rhizosphere
microbiome**

Abstract

The use of urea fertilizer must be accompanied of good soil management practices, otherwise it will cause serious environmental problems due to nitrogen losses via nitrate leaching and/or ammonia volatilization to the atmosphere. Ammonia volatilization comes from the hydrolysis of urea when applied to the soil surface for agricultural purposes. Urease inhibitors are a technology that delays the hydrolysis of urea on soil surface, allowing the fertilizer to reach the rhizosphere for its successful hydrolysis to promptly provide inorganic N to plants to. In this work, *Cenchrus americanus* plants were cultivated for 35 days in a greenhouse in pots containing latossolo vermelho distrófico soil, in the presence of urea (150 mg of N/Kg of soil) supplemented or not with two new urease inhibitors derived from amino acid D-tryptophan (D-106 and D-Ac-106) or NBPT at 0.06% or 1% (w/w). Both inhibitors D-106 and D-Ac-106, opposed to NBPT, promoted an increase in root biomass in relation to plants treated only with urea. At the end of the experiment, D-tryptophan derivatives also promoted greater accumulation of important nutrients such as nitrogen, phosphorus, sulfur, among others in plant shoots. D-Ac-106 (0.06%) and NBPT (1%) reduced the (Operational Taxonomic Unit) OTU abundance and OTU richness estimated by the Chao1 index 24 h after treatment application. While D-Ac-106 promoted changes in the microbiome composition throughout all evaluated time, D-106 changed the composition only on the 35th day. Amino acid-derived (thio)hydantoins, despite bringing benefits to plant development, negatively affected the bacterial microbiota, changing the taxon composition, reducing connectivity between modules and modifying the topology of the microbial network of millet rhizosphere.

Keywords: Microbial community, Mineral nutrition, Urea fertilization, Urease inhibitors.

1. Introduction

The use of nitrogen fertilizers has considerably increased in recent years (Liu et al., 2022). One of the fertilizers most used worldwide to ensure high yields is urea. However, its use is associated with environmental pollution, due to its rapid hydrolysis on the soil surface by soil urease to yield the gasses ammonia and carbon dioxide (Tavares, et al., 2021). In addition, high doses of urea are associated with degradation and loss of soil quality (Fox, 2004). The use of urease inhibitors is one of the strategies adopted to mitigate N losses from urea (Gao et al., 2015). The commercial inhibitor *N*-(butyl)thiophosphoric triamide (NBPT) is the urease inhibitor most used in agriculture. New urease inhibitors have been extensively researched, since NBPT has some disadvantages such as poor storage stability and rapid degradation in acidic soils (Gholivand et al., Islam et al, 2019).

(Thio)hydantoins are a new class of compounds capable of inhibiting urease in *in vitro* tests (Camargo et al., 2022). These molecules are naturally produced by the roots of some species from the *Brassicaceae* family, such as *Armoracia rusticana* and *Lepidium meyenii*. The effect of (thio)hydantoins on plants and soil is completely unknown.

Soil is the habitat with the greatest diversity of microorganisms on the planet, with the greatest diversity being bacteria (Mendes et al., 2013). The greater activity of these microorganisms in the soil depends on constant sources of nutrients, since soils can be compared to large nutritional desserts, in addition to being an environment of extreme competition. Therefore, greater biological activity is found in the rhizosphere, since roots are able to release exudates, which constitute a rich nutritional source that is readily consumed by microorganisms (Pii et al., 2015). This is one of the reasons by which the rhizosphere is such a peculiar place, due to the fine contact between plants and soil, where a wide variety of important interactions is established (Pérez-Izquierdo et al., 2019). In rhizosphere, microorganisms, releasing phytohormones that improve plant performance. In addition, they fix nitrogen and solubilize nutrients such as phosphorus, potassium and iron, making them more available for uptake by plants (Yadav et al., 2021).

Some studies have shown that NBPT can alter the structure of the prokaryotic community in the soil (Li et al., 2019) and the abundance of functional groups such as those related to ammonia oxidizing bacteria (Dong et al., 2018; Fan et al., 2018). Here

it was evaluated the effect of NBPT and new amino acid-derived urease inhibitors on the rhizosphere microbiome, as well as on the nutrition of *Cenchrus americanus* grown under controlled conditions.

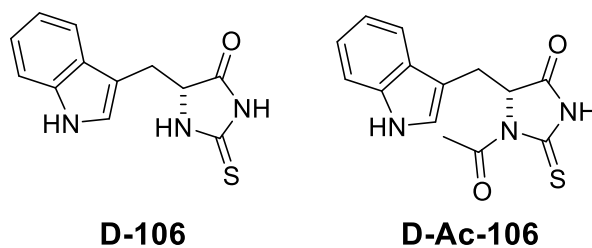


Figure 1. Structures of acetyl (thio)hydantoin and thiohydantoin.

2. Materials and methods

2.1 Soil ureases inhibition

Soil urease inhibition by (thio)hydantoin D-106 and D-Ac-106 was carried out with samples of a latossolo vermelho distrófico soil. For that 0.5 g of soil was incubated at 37°C for 1 h with 72 mM urea by itself or in combination with each inhibitor at a final concentration of 500 µM. The NBPT was used as a reference. Reactions were stopped by adding a solution of 1 M KCl in 10 mM HCl, followed by incubation at 25 °C for 30 min. After centrifugation, a fraction of the supernatant was collected and added to a solution containing 3.4% w/v sodium salicylate, 2.5% w/v sodium citrate, 2.5% w/v sodium tartrate and sodium nitroprusside. 1.2×10^{-4} % m/v. The mixtures were then incubated at 25 °C and 600 rpm in the dark for 15 min followed by addition of 3% w/v sodium hydroxide and 1% v/v sodium hypochlorite. The systems were incubated at 25 °C and 600 rpm in the dark for 1 h and analyzed at 660 nm to quantify the NH_4^+ formed (Kandeler and Gerber, 1988).

2.2 Greenhouse experimental design

Soil was collected at Embrapa Milho and Sorghum in Sete Lagoas Minas Gerais (19° 28 '01.2 "S and 44° 10' 24.5"W) in the 0 to 20 cm layer. The soil type was Latosolo Vermelho Distrófico, the physical and chemical characteristics of the soil were 57.5 % sand + silt, 42.5% clay, pH(H₂O) 6.3, N 0.171 dag kg⁻¹, P(Mehlich) 26.3 mg dm⁻³, K(Mehlich) 219 mg dm⁻³, Ca²⁺ 4.8 cmol_c dm⁻³, Mg²⁺ 1.1 cmol_c dm⁻³, Al absent, H⁺ Al 3.9 cmol_c dm⁻³, organic matter 2.4 dag dm³ and 6.18 % base saturation.

The experiment was completely randomized with four replications each, including four treatments supplemented with urea (150mg of N/Kg of soil) and urease inhibitors (D-106, D-Ac-106 and NBPT) at 0.06% or 1% w/w. Treatments containing urea only and a blank control constituted of the solubilizing solution were also used. The treatments were applied in pots containing 4 kg of soil and 4 millet seedlings (14 day-old from the sowing). The compounds were applied as solutions to ensure their homogeneous distribution in the soil. Compounds were solubilized in a solution composed of 0.015% ethanol, 0.015% DMSO and 0.5% polyethylene glycol 400. For sowing, each pot received 10 millet seeds and thinning was performed, leaving only the plants with uniform growth. The soil was maintained at 75 % of field capacity

throughout the experiment. After 1, 3 and 35 days the plants were removed from the pot, the soil excess loosely bound to the roots was shaken off and the soil tightly adhering to the roots was brushed off in a sterile plastic bag. Afterwards, aliquots were placed in liquid nitrogen until further analyses. Thus, the sample was defined as a mixture of rhizospheric soil from the 4 plants present in the same pot. At the end of the experiment, the plants were weighed to obtain the fresh mass of the aerial part and the root, later they were placed in an oven with forced air circulation (65°) to obtain the dry mass. The dried plants were ground in a Willye R-TE-650/1 mill and the nutrient content was quantified.

2.3 DNA extraction and sequencing

DNA extraction was done using the FastDNA™ SPIN Kit for Soil (MPBiomedicals) according to the manufacturer's recommendations. After extraction, DNA quality was assessed by the ratios of 260/280 nm using a Thermo Scientific™ NanoDrop™ Lite and electrophoresis.

2.4 DNA Sequencing and amplicon data analysis

Amplification of the V3-V4 region of the 16S rRNA gene was performed with primers 341F (forward) and 806R (reverse). Libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., USA). The pipeline for analysis of 16S amplicons was used, whose source code is deposited on GitHub: <https://github.com/LBMCF/pipeline-for-amplicon-analysis>. The pipeline is cross-platform and was developed in Python 3, allowing the analysis of the 16S amplicon readings using OTU approaches, returning a table of OTU abundances based on the SILVA SSU NR 138.1 database (Quast et al., 2012). The pipeline uses the programs: 32-bit USEARCH (Edgar, 2010), VSEARCH (Rognes et al., 2016), Cutadapt (Martin, 2011), FastQC (Andrews, 2010) and BLAST (Altschul et al., 1990).

An evaluation of the quality control of the readings was carried out using FastQC, it merges all the direct and reverse readings through VSEARCH with the `fastq_mergepairs` option. VSEARCH was also used for quality control and elimination of duplicates and chimeras. Cutting of adapters was performed with Cutadapt. The grouping of readings in OTUs was performed using VSEARCH with 97% identity, for

taxonomic identification the BLASTn module of BLAST was used with the SILVA database.

2.5 Microbiome composition, diversity and Tax4Fun prediction

All composition and alpha and beta diversity analyses were performed on the Microbiome Analysis platform (<https://www.microbiomeanalyst.ca>). For this, the data were log-centered transformed. TAX4Fun predictions were also performed on the platform. To assess differentially expressed pathways between treatments, each treatment was compared with the control (urea treatment without inhibitors) at each time point. The differentially expressed pathways between treatments were identified and each component (KEGG CODE) was analyzed in the STAMP program. Correlations of microbiome composition with data on nutrient accumulation were performed with canonical correlation analysis using the platform using Metagenomics for Environmental Microbiology (MEM) (<http://mem.rcees.ac.cn:8080/>).

2.6 Bacterial community structure

The ecological association networks based on (Operational Taxonomic Unit) OTUs correlation at the end of the experiment, in order to compare the treatment networks between D-106, D-Ac-106 and NBPT, were built according to the random matrix theory (RMT) approach, elaborated in the Analysis pipeline of Molecular Ecological Network (MENA) available at <http://ieg4.rccc.ou.edu/mena> (Deng et al., 2012). For this, the abundance tables with the concentrations 0.06% and 1% formed a total of 8 replicates for RMT analysis. The RMT approach is more objective, since the eigenvalue of correlation matrices is not defined empirically, but when there is a change in the Wigner-Dyson to Poisson statistic (Deng et al., 2012). The OTUs with more than 10 readings and present in more than 60% of the samples were subjected to correlation analysis using Pearson's correlation approach in a take logarithm abundance table. The network was built using correlation limits determined by the RMT and the fast greedy modularity optimization method (Clauset et al., 2004). The topological properties of the empirical molecular ecological network (MEN) were calculated and compared to verify whether the treatment with inhibitors affects the profiles of modules and network interactions. Mantel and eigengene analysis were

performed to assess whether modules are associated with nutrient accumulation. The networks were visualized in Cytoscape (v. 3.6.1) (Lotia et al., 2013).

2.7 Statistical analysis

The analyses of the urease inhibition and millet growth data were done in the R program (V 4.2.1) using ScottKnott clustering at significance of 0.05. Beta diversity was analyzed by permanova and alpha diversity by t test between treatment and control with urea (Inhibitor-free), all with a significance level of 0.05. Empirically obtained topological properties were analyzed by anova, using the standard deviation obtained from the radomic matrices.

3. Results

3.1. Urease soil inhibition, biomass and nutrients accumulation

Both compounds inhibited the soil ureases at the same intensity as NBPT (40 to 60%) (Figure 2). In addition, both compounds stimulated the accumulation of biomass in the root system, without compromising the accumulation of biomass in the shoot (Figure 2C and D). The tryptophan derivative D (1%) stimulated the accumulation of N, P and K in the shoot, while the compound at 0.06% promoted the accumulation of Fe in the shoot and Cu in the root. On the other hand, the acetylated Trp derivative stimulated the accumulation of N, P, Mg, S, Mg, Cu and Zn in the shoot. NBPT at 1% stimulated the accumulation of Fe in the shoot (Figure 3).

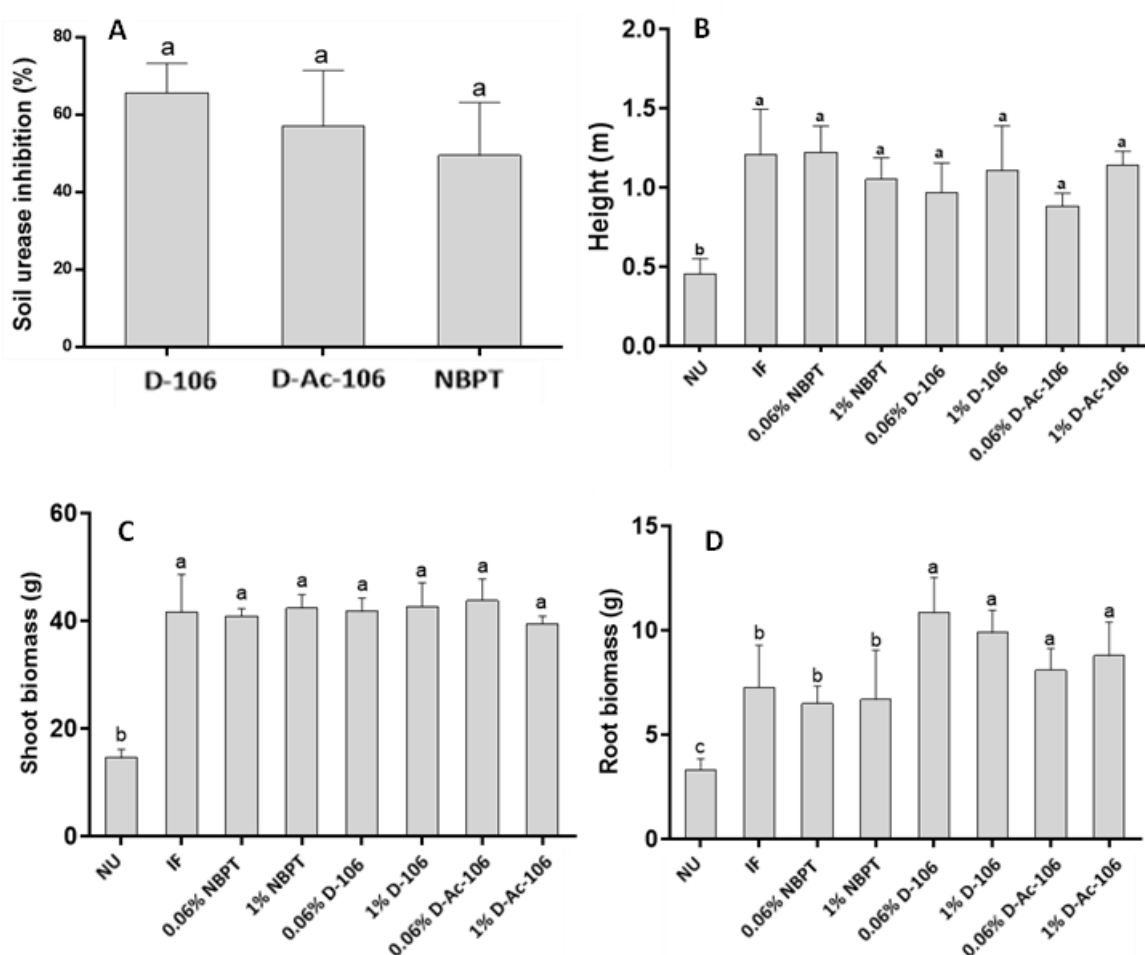


Figure 2. Effect of (thio)hydantoin derivatives from amino acid on soil urease inhibition, compounds at 500 μ M (A). Values correspond to the mean $\pm$ standard deviation (n=3). Effect of new urease inhibitors on height (B) biomass accumulation in shoot (C) and root system (D)

after 35 days of treatment. Values correspond to the mean $\pm$ standard deviation (n=4). Values followed by distinct letters are significantly different from each other by the Scott-Knott test ($P > 0.05$).

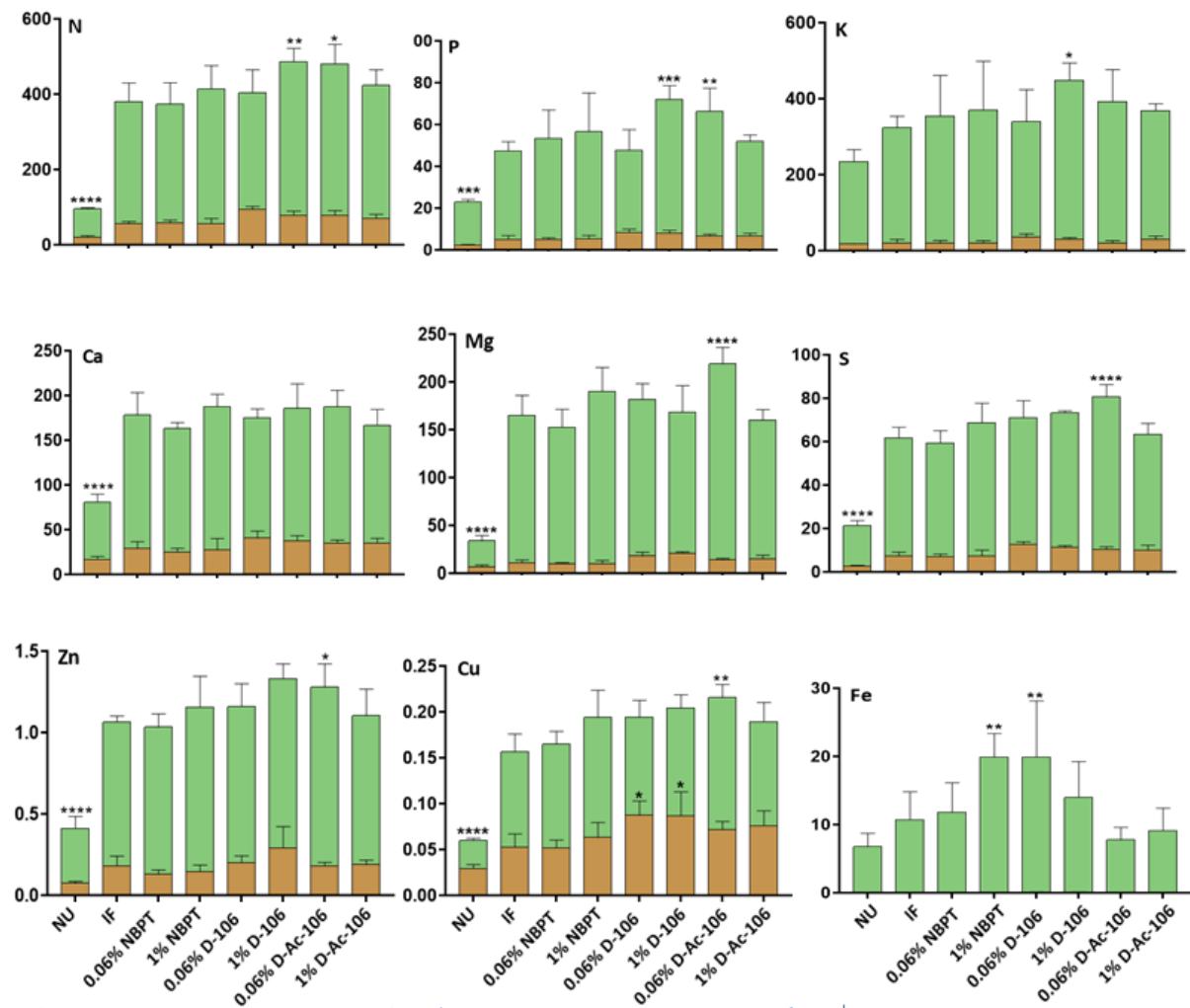


Figure 3. Accumulated nutrients (mg.plant⁻¹) in the shoot (green) and root (brown) of millet after 35 days of treatment. Values represent mean $\pm$ standard deviation (n=4). Asterisks correspond to the level of significance by the ANOVA test ($p < 0.05$) $P < 0.033$ (*); 0.021 (**); 0.002 (***); 0.0001 (****) followed by Dunnett's multiple comparison test. All treatment was compared with the control (IF).

3.2 Microbiome changes

The most abundant microbial genera in the rhizosphere were *Sphingomonas*, *Bradyrhizobium*, *Chitinophaga*, *Mesorhizobium*; several other genera could not be classified (Figure 3). Treatment with urea promoted an increase in the abundance of *Ralstonia* and *Cupriavidus* (Figure 4 and Figure S1). The greatest change in the composition of the microbiome was observed along the time of soil treatment, with an evident difference between the initial microbiome (1st and 3rd day) and that after 35 days (Figure 5B). Microbiome composition was affected by all urease inhibitors. The NBPT at 1% significantly altered the microbiome composition on the first and third day after treatment (Figure S2). NBPT 1% also reduced abundance and Chao1 diversity index 24 h after treatment (Figure S2). The D-Ac-106 inhibitor promoted microbiome modifications along the time course. Within 24 h, the inhibitor at 0.06% modified the microbiome composition (Figure S2) and reduced species abundance and richness (Figure S3). On the third day, the composition of the microbiome was still altered by the presence of the inhibitor. (Thio)hydantoin D changed the composition of the microbiome only on the 35th day after the treatments were applied (Figure 5A). At the end of the experiment the D-Ac-106 treatment was the most distant composition in relation to the urea treatment. NBPT did not alter the microbiome and compound D-106 appeared as an intermediate between urea and D-Ac-106 treatment (Figure 5A).

On the 3rd day, D-Ac-106 at 0.06% and 1% and D-106 1% reduced the abundance of the genus *Aminobacter*. D-Ac-106 at 1% increased the abundance of the genera *Luteolibacter*, *Amycolatopsis*, *Edaphobaculum* and *Chitinophaga*. At the end of the experiment, NBPT, regardless of the concentration tested, increased the abundance of the taxa classified as wastewater_metagenome. D-Ac-106, regardless of the concentration tested, reduced the abundance of the genera *Acidicaldus*, *Alsobacter*, wastewater_metagenome and *Planotetraspora* while D-Ac-106 at 1% reduced the abundance of the genera *Acidiphaera*, *Armatimonadetes* and *Luteimonas*. (Thio)hydantoin D-106 at 0.06% decreased the abundance of the genera Wastewater_metagenome and *Azospirillum* and at 1% reduced the genera wastewater_metagenome and *Oryzihumus* while increasing the abundance of the genera *Coxiella* (Table S1).

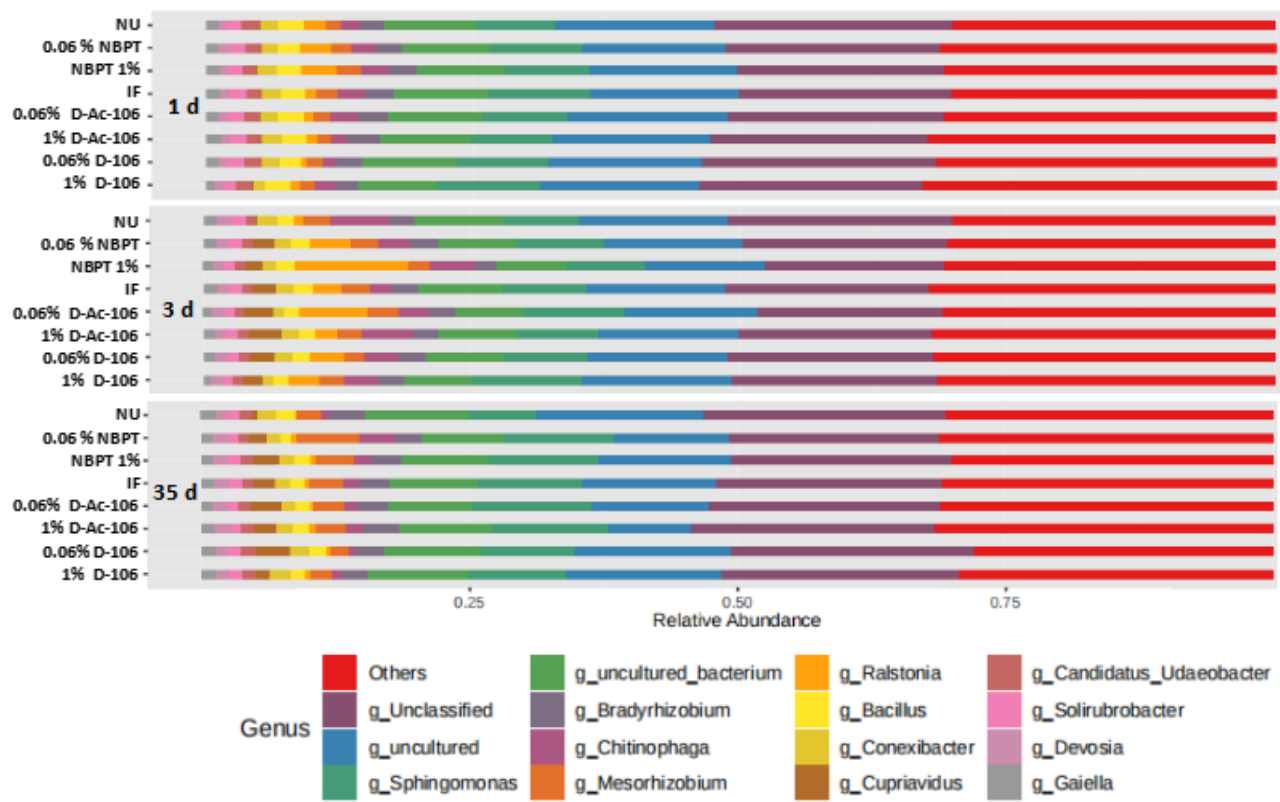


Figure 4. Percentage of abundance of the 15 most common genera in millet rhizosphere cultivated in latossolo vermelho distrófico soil.

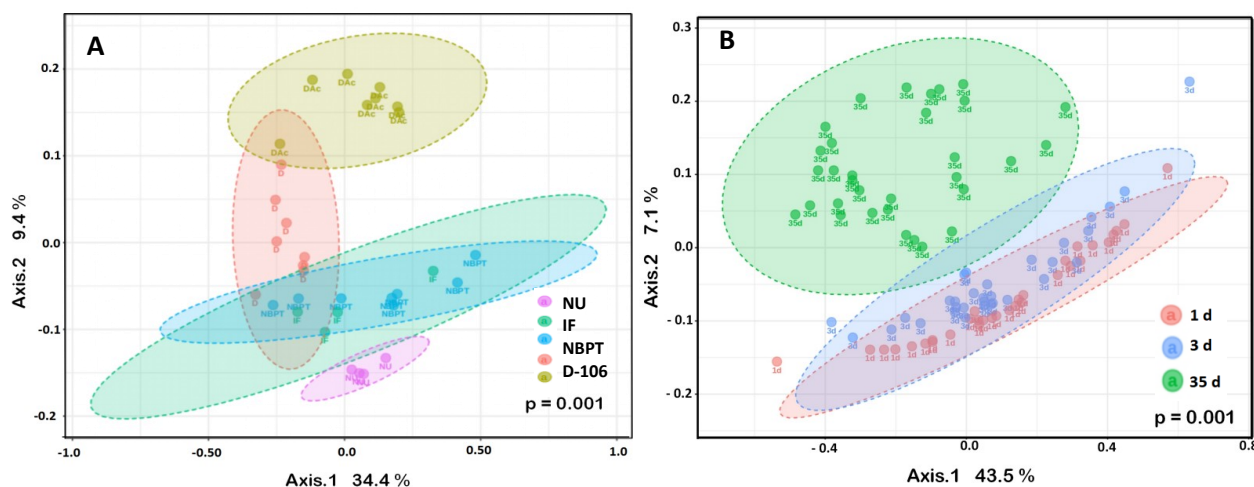


Figure 5. A- PCoA of Pearl millet bacterial rhizosphere community based on Bray Cutis distance. All treatment on the 35th day. **B** - Time effect on microbial composition.

3.3 Microbiome functional prediction Tax4Fun

The main pathways present in the millet rhizosphere soil were those related to the metabolism of amino acids, carbohydrates and energy (Figure S5). Significant differences in routes were present only at the end of the experiment (Figure S6), between treatment without urea vs urea (Table S2) and D-Ac-106 vs urea (Table S3). D-Ac-106 treatment stimulated pathways related to xenobiotic degradation (Table S3).

3.4 Molecular network structure

After pre-processing the OTU abundance table, treatment with NBPT had the lowest number of OTUs (587), while (thio)hydantoin D-106 treatment had the highest number (1331). Thresholds (s^t) were very similar among the treatments - NBPT (0.98), D-106 (0.96), D-Ac-106 (0.97) and were applied for construction of each network (Table 2). There was a statistical difference between the three indices obtained to describe network properties: modularity, which measures the capacity of the network to form clearly delimited modules; geodesic distance, which is the shortest path between two nodes, and the clustering coefficient, which means the connection of a node with other neighboring nodes. Thus, while (thio)hydantoin D-106 led to greater modularity and lower geodesic distance between nodes, this compound promoted less

ability for nodes to connect to each other (Table 2). The size of modules and links were considerably reduced by the (thio)hydantoins treatment, despite the greater number of modules. Both (thio)hydantoins resulted in a similar configuration of modules, two large modules with over 70 nodes and several smaller modules. As for the NBPT treatment, there were 4 larger modules with more than 80 nodes and more than twice as many links present in the D and D-Ac-106 network (Table 2; Figure 7). *Actinobacteriota* and *Proteobacteria* were the most abundant phyla in the nodes. The *Firmicutes* and *Myxococcota* phylum was proportionally lower in treatments with (thio)hydantoins in comparison to the control (Figure 8). The (thio)hydantoins modified the high hierarchical organization between the module pairs, compared to the NBPT treatment. While the NBPT treatment produced modules with high interconnection, this strong correlation was reduced in the D-Ac-106 treatment and even more so in the D-106 treatment (supplementary S5, S6 and S7).

3.5 Topological properties

Topological configurations were also very different between treatments (Figure S8). This classification is based on the connectivity parameters within (Z_i) and between (P_i) the modules. Thus, nodes with little connectivity within their module and between other modules are considered peripheral nodes. Nodes that have multiple links with other modules (high P_i) and low Z_i are classified as connectors. Modular hubs, on the other hand, have high Z_i and low P_i , while network hubs have high Z_i and P_i values (Zhou et al., 2011; Olesen et al., 2007). In the NBPT treatment, 95.7% of the OTUs were peripheral nodes. 3.8% of the nodes were classified as connectors and 0.5% as module hubs. In the D-Ac-106 treatment, 96.3% of the nodes were peripheral, 3.1% of the nodes were classified as connectors and 0.6% were module hubs. In the treatment with D-106, 97.0% of the nodes were peripheral, 1.7% of the nodes were classified as connectors and 1.2% were module hubs. In none of the three treatments were network hubs observed (Figure S10; Table S4). In the treatment with NBPT, the most abundant connectors were from the phyla *Acidobacteriota* (25%) and *Actinobacteriota* (25%). The hub modules include *Actinobacteria* and *Gemmatimonadota*. The Compound D-106 reduced the proportion of connectors and increased the proportion of hub modules. In addition, it changed the affiliation of key organisms, as the most abundant phyla of connectors were *Proteobacteria* (40%) and module hubs *Proteobacteria*

(28.5%) and *Actinobacteriota* (28.5%). The D-Ac-106, on the other hand, maintained the same proportions of key microorganisms in the topological configurations as NBPT. However there was a great difference in the taxa of these organisms. The main connector was *Chloroflexi* (45%) and the module hubs were *Actinobacteriota* and *Chloroflexi* (Table S4).

Table 1. Topological properties of molecular ecological networks (MENs) of microbial communities as function of soil treatment with urease inhibitors.

Network	Index	NBPT	D-106	D-Ac-106
	^a Number of OTUs	587	1331	680
	S ^t	0.98	0.96	0.97
	^b Number of nodes	422	576	354
	Number of edges (average connectivity)	3197 (15.15)	1020 (3.54)	1430 (8.07)
	Connectedness (Con)	0.37	0.38	0.64
	Power-law R ²	0.92	0.99	0.97
	Power law (free-scale) R ²	0.77	0.91	0.83
	Exponential distributions (faster-decaying functions) R ²	0.98	0.96	0.93
	Truncated power-law (broad scale) R ²	0.40	0.80	0.53
	Modularity (N° of modules)	0.38 (22)****	0.68 (91)****	0.48 (40)****
^c Empirical	Average clustering coefficient (C)	0.44 ****	0.20 ****	0.34 ****
	Harmonic geodesic distance (HD)	2.48 ****	4.59****	3.23 ****
	Average modularity ± SD	0.176 ± 0.004	0.55 ± 0.006	0.292 ± 0.005
^d Random	Average clustering coefficient (Cr) ± SD	0.074 ± 0.007	0.004 ± 0.002	0.029 ± 0.006
	Harmonic geodesic distance (Lr) ± SD	2.479 ± 0.013	4.029 ± 0.037	2.8 ± 0.019

^a The number of OTUs used for network construction.

S^t similarity threshold

^bThe number of OTUs (i.e., nodes) in a network.

^c Empirical networks

^d The random networks were generated by 100 reconnections of the links of a pMEN with the identical number of nodes and links.

**** statistical significance (P < 0.001).

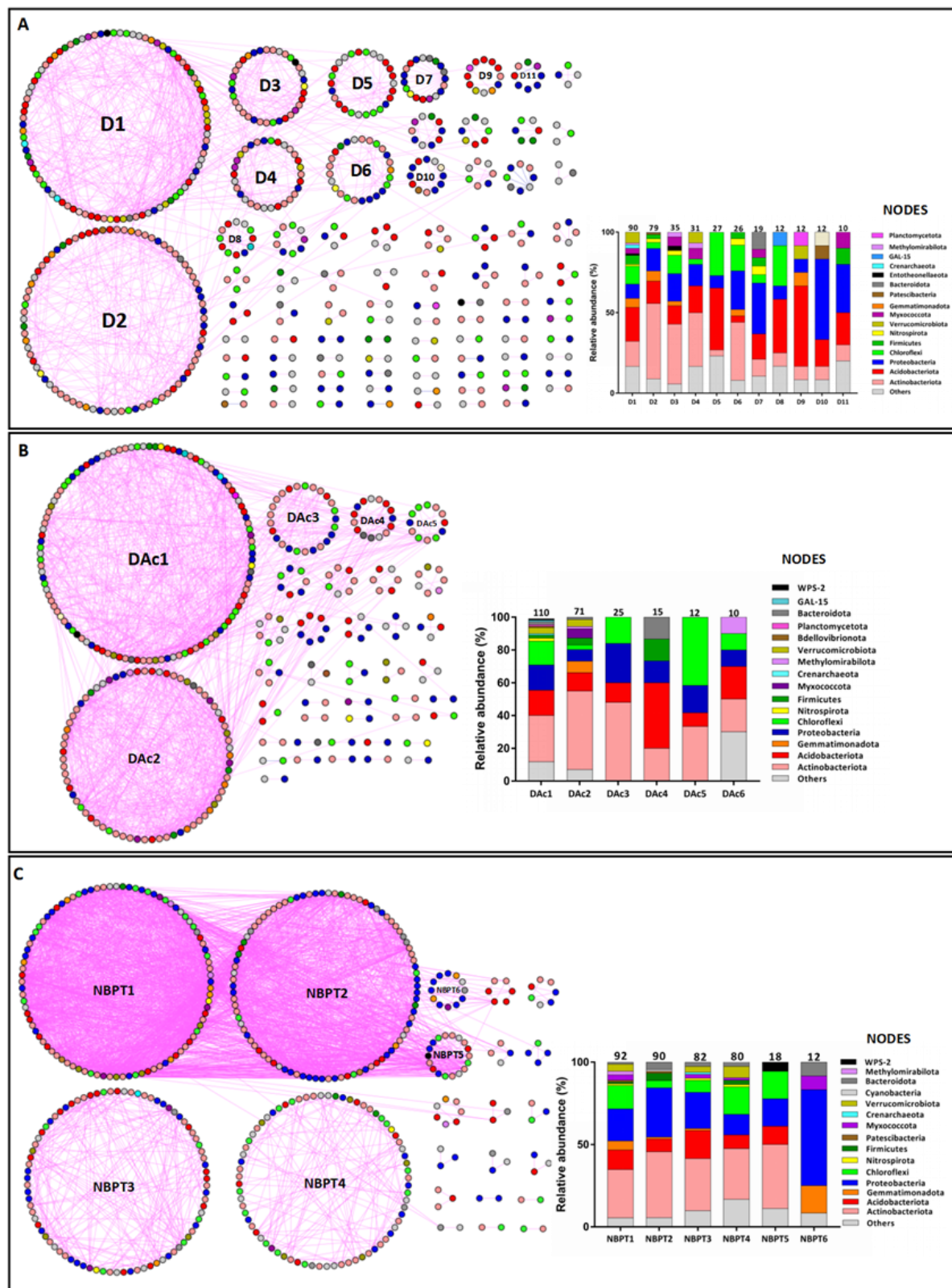


Figure 7. The network in pearl millet rhizosphere soil 15 days after treatment with D-106 **(A)**, D-Ac-106 **(B)** and NBPT **(C)**. On the right, a histogram with the relative abundance of each phylum and the number of nodes in each module. The color of each node was determined by

phylum. The pink edges represent positive correlations between two nodes, while darkblue edges represent negative correlations.

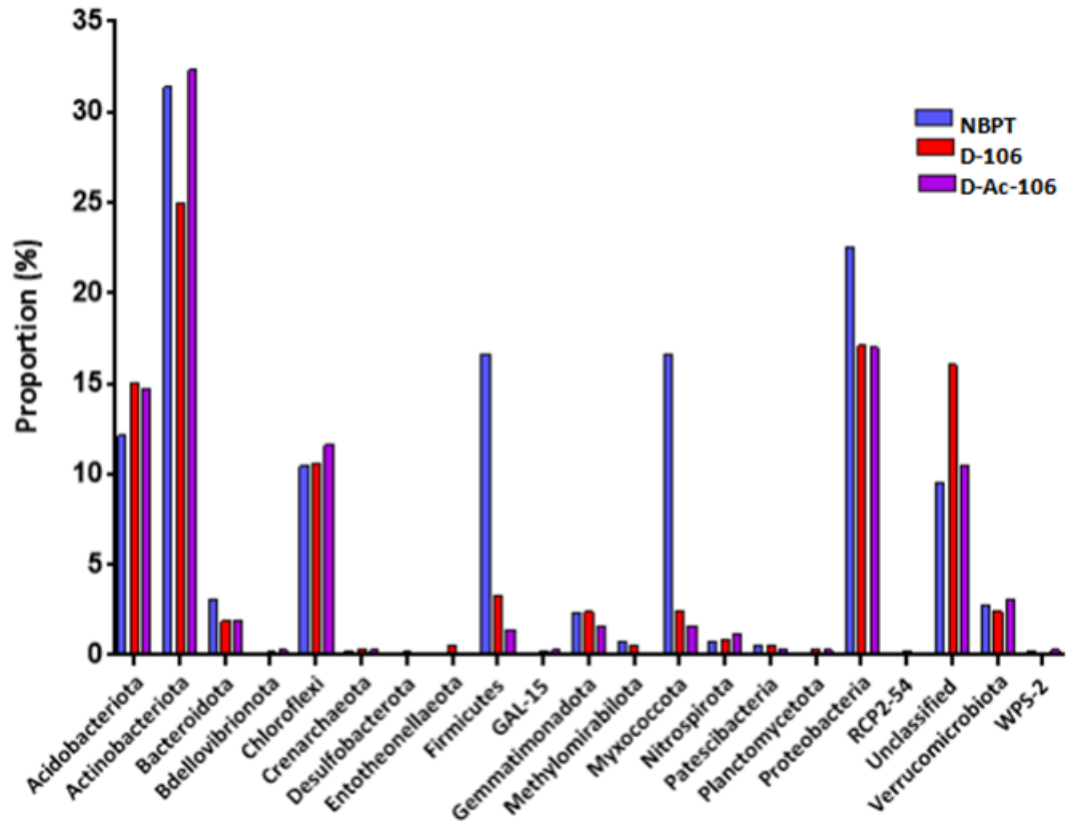


Figure 8. Relative abundances of different nodes in NBPT, D-106 and D-Ac-106 networks.

4. Discussion

Some compounds are known to promote biomass accumulation in roots. Examples are some amino acids such as glycine (Zobiolo et al., 2012; Aghaye Noroozlo et al., 2019), fulvic acid (Rauthan and Schnitzer 1981) and the production of the phytohormone indole-3-acetic acid (IAA) by rhizosphere-associated bacteria (Fierro-coronado et al., 2014). (Thio)hydantoins acted as plant biostimulants, which are substances or microorganisms capable of promoting an increase in plant biomass and increasing nutrient uptake capacity (Vessey, 2003).

Although D-106 and D-Ac-106 had a similar effect regarding the increase in root biomass and the accumulation of some nutrients, the effect on the microbiome was not similar regarding their composition at the end of the experiment (35 days post-treatment). This indicates the acetylation of D-106 contributes to the differential bioactivity of D-Ac-106 also related to sulfur and magnesium uptake by roots. Other events not investigated here, such as root growth and greater soil exploration capacity, as well as greater mycorrhiza activity, may also be related. Functional prediction by TAX4FUN did not show greater abundance of any known genes related to enzymes that act in the availability of nutrients in the soil (data not shown).

The use of amino acids such as glutamate, glycine in the soil has enabled a higher concentration of both macro (K, Mg, Ca) and micronutrients (Fe and Zn) in plants (Aghaye Noroozlo et al., 2019) due to the stimulation of the nutrients uptake and their translocation to plant shoots (Sh Sadak et al., 2015). The greater accumulation of Mg, Fe and Zn may also be related to the acidification promoted by these reduced forms applied in the rhizosphere and, consequently, greater availability of these nutrients (Aghaye Noroozlo et al., 2019). Here it was demonstrated that the greater accumulation of nutrients in millet treated with tryptophan derivatives is strongly related to the alteration of the microbiome composition. The application of L-tryptophan in the soil promotes plants' growth (Mohite, 2013), mainly due to its conversion to IAA by growth-promoting rhizobacteria (Sasirekha et al., 2012).

The (thio)hydantoins, regardless of the concentrations tested, promoted changes in the microbiome that were accompanied by an increase in the accumulation of nutrients in millet. The reduction in the abundance of microorganisms 24 h after the application of the treatments may lead to the perception that the absorbed nutrients came from the microbial biomass. However, (thio)hydantoin D-106 did not promote this

reduction, but stimulated the accumulation of N and P (Figure 3). One hypothesis is that the bioactive (thio)hydantoin is acetylated (D-Ac-106) where at low concentrations it is able to exert its effect quickly in the soil. Compound D, in turn, may be slowly transformed in the soil into its acetylated bioactive form (Zhang et al., 2014). As it is transformed slowly, it allows the microbiota to adapt to its presence in the soil.

The rhizosphere microbiome is shaped by several factors such as plant cultivar type and genotype (Bugarelli et al., 2015), and phenology as well (Xiong et al., 2021). One of the main factors of change in the microbiome, in addition to treatment with inhibitors, was time (Figure 5B). In fact, the stage of plant development is intrinsically related to both the composition and the ecological functions of the microbiome (Chaparro et al., 2014; Xiong et al., 2021). In addition to the different composition, the development of pearl millet favored an increase in the diversity of the microbiome (Figure S3). Diversity is an important factor related to microbiome resilience (Feng et al., 2017).

However, (thio)hydantoins modified the microbiome over the time in a concentration-dependent manner. The acetylated (thio)hydantoin that promoted changes in the beginning (Figure S2) and at the end of the experiment (Figure 4A). Despite the very similar molecular structure between the two (thio)hydantoins, at the end of the experiment the microbiota compositions were significantly different between these treatments ($p = 0.001$). The acetylation of the Trp derivative makes the molecule more structurally similar to IAA one, which also acts at very low concentrations in the soil (Szajdak and Maryganova, 2007). It is known that one of the effects of IAA is the inhibition of antibiotics biosynthesis, which is an important competitive microbiota tool (Matilla et al., 2018). The application of the hormone gibberellin with an urease inhibitor (NBPT) was able to increase the N uptake in maize, by increasing cell division of roots (Ghodrat et al., 2012; Ullha et al. a., 2022). Thus, D-Ac-106 seems to have promoted some toxicity to the microbiota. At the end of the experiment, despite lower abundance and richness, this difference was not significant (Figure S2). On the other hand, the microbiota adapted to the presence of (thio)hydantoin D, since it promoted changes in the microbiota without altering the diversity of and species richness.

Both (thio)hydantoins reduced the genus *Aminobacter* in the first days after application of the treatments. In other works, strains of this genus have demonstrated an enormous capacity to promote plant growth (Velasco-Jiménez et al., 2020). Furthermore, it has been shown to be a genus that is quite sensitive to the presence

of contaminants such as heavy metals (Li et al., 2022). However, on the 3rd day D-Ac-106 (1%) was able to increase the levels of important genera related to soil repair such as *Luteolibacter* (Wu et al., 2021).

Contrasting effects of NBPT on the microbiome have been reported in the literature. Several works have shown that the use of NBPT affects the composition and abundance of bacteria and archaea in the soil microbiome (Li et al., 2019; Castellano-Hinojosa et al., 2020). Others show that NBPT has no effect on the edaphic microbiome (Fu et al., 2020; Duff et al., 2022). In the present work, the NBPT at 1% reduced the abundance and diversity at the beginning of the experiment, a condition that was restored to the basal levels at the end of the experiment. This could be both due to the high resilience of the microbiome and the relatively rapid degradation of NBPT in soil (Engel et al., 2015; Peters et al., 2022).

Several works have carried out studies based on RMT-network in order to assess the impact of environmental changes or the effect of pollutants on the structure of microbial communities (Zhou et al., 2011; Chen et al., 2018). This approach is very interesting since little can be predicted just by evaluating the changes in certain taxonomic lineages. This is due to functional redundancy, often changes may not significantly alter the functions and interactions present in the original microbiome. The use of this approach can elucidate, for example, the impact of CO₂ on the structure of the soil microbiome (Zhou et al., 2011) and of stressors on aquatic communities (Chen et al., 2018). The number of nodes, as well as the average connectivity are indexes used to predict the complexity of a network (Zhou et al., 2010). When comparing healthy soil (HS) and a bacterial wilt-susceptible soil (BWS) Qi et al., (2019) demonstrated how the interactions and number of nodes and modules were drastically reduced in BWS networks compared to HS. The number of nodes and edges were also drastically reduced in soils contaminated with heavy metals (Li et al., 2017). The modules are formed by nodes that are much more connected to each other than to other nodes and can be related to ecological niches (Zhou et al., 2011). All indexes obtained here are in accordance with the characteristics that describe small-world networks (Watts & Strogatz 1998). Both (thio)hydantoin promoted changes in the structure of the networks and greatly increased the number of modules in relation to the NBPT. However, they may have left the microbiome structure more vulnerable, by reducing the number of interactions and generalist connecting key taxa (Wang et al., 2018). They also significantly reduced the number of links between modules. However,

both (thio)hydantoins increased the number of modules, and D-106 also increased the number of nodes. In maize rhizospheric soils contaminated with lindane, the presence of the pollutant increased both the number of nodes and the connectivity between nodes (Feng et al., 2020). Karime et al, (2019) demonstrated that increased network fragmentation and reduced network interactions, which reduced complexity, occur with increasing intensification of agricultural activity, despite increased biodiversity.

5. Conclusion

The application of (thio)hydantoins in the soil promoted an increase in the performance of pearl millet cultivated for 55 days in pots with latossolo vermelho distrófico soil. Both compounds promoted an increase in root biomass and, at the same time, a greater accumulation of nutrients. However, these compounds showed different effects on the composition and structure of the microbiome likely due to the presence of an acetyl group in D-Ac-106. While acetylated (thio)hydantoin (D-Ac-106) promoted loss of abundance and diversity, compound D-106 did not negatively affect these microbiome traits. Both (thio)hydantoins promoted a restructuring of the microbiome, by increasing the number of modules and reducing the total number of links of the microbial network. D-Ac-106 stimulated routes of xenobiotics degradation and the accumulation of nutrients could be derived from the microbial biomass in the soil. More studies are needed to investigate a possible signaling role of this compound. (Thio)hydantoin D, on the other hand, did not affect the function, nor the diversity and richness of the microbiome. However, it also promoted network fragmentation and greatly reduced modular interactions. This work demonstrates that increased plant performance is not always associated with improved robustness and health of the microbiome.

References

- Aghaye Noroozlo, Y., Souri, M. K., & Delshad, M. (2019). Effects of soil application of amino acids, ammonium, and nitrate on nutrient accumulation and growth characteristics of sweet basil. *Communications in Soil Science and Plant Analysis*, 50(22), 2864-2872.
- Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Weiman, A., Dröge, J., ... Schulze-Lefert, P. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host and Microbe*, 17(3), 392– 403.
- Camargo, P. G., Fabris, M., Nakamae, M. Y. T., de Freitas Oliveira, B. G., da Silva Lima, C. H., de Fátima, Â., ... & Macedo Jr, F. (2022). (thio)hydantoin and hydantoin derived from amino acids as potent urease inhibitors: Inhibitory activity and ligand-target interactions. *Chemico-Biological Interactions*, 365, 110045.
- Castellano-Hinojosa, A., Gonzalez-Lopez, J., Vallejo, A., & Bedmar, E. J. (2020). Effect of urease and nitrification inhibitors on ammonia volatilization and abundance of N-cycling genes in an agricultural soil. *Journal of Plant Nutrition and Soil Science*, 183(1), 99-109.
- Chen, W., Wilkes, G., Khan, I. U., Pintar, K. D., Thomas, J. L., Lévesque, C. A., ... & Lapen, D. R. (2018). Aquatic bacterial communities associated with land use and environmental factors in agricultural landscapes using a metabarcoding approach. *Frontiers in Microbiology*, 9, 2301.
- Clauset A, Newman ME, and Moore C. (2004). Finding community structure in very large networks. *Phys. Rev. E Stat. Nonlin. Soft Matter Phys.* 70:066111.
- de Carvalho, P. G., Ribeiro, J. M., Garbin, R. P. B., Nakazato, G., Yamada Ogatta, S. F., de Fátima, Â., ... & Macedo, F. (2020). Synthesis and antimicrobial activity of (thio)hydantoin obtained from L-amino acids. *Letters in Drug Design & Discovery*, 17(1), 94-102.
- Deng, Y., Jiang, Y.-H., Yang, Y., He, Z., Luo, F., and Zhou, J. (2012). Molecular ecological network analyses. *BMC Bioinformatics* 13:113. doi: 10.1186/1471-2105-13-113
- Buchynskyy, A., Gillespie, J. R., Herbst, Z. M., Ranade, R. M., Buckner, F. S., & Gelb, M. H. (2017). 1-Benzyl-3-aryl-2-(thio)hydantoin derivatives as new anti-*Trypanosoma brucei* agents: SAR and in vivo efficacy. *ACS medicinal chemistry letters*, 8(8), 886-891.

- Dong, D., Kou, Y., Yang, W., Chen, G., & Xu, H. (2018). Effects of urease and nitrification inhibitors on nitrous oxide emissions and nitrifying/denitrifying microbial communities in a rainfed maize soil: A 6-year field observation. *Soil and Tillage Research*, 180, 82-90.
- Duff, A. M., Forrester, P., Ikoyi, I., & Brennan, F. (2022). Assessing the long-term impact of urease and nitrification inhibitor use on microbial community composition, diversity and function in grassland soil. *Soil Biology and Biochemistry*, 108709.
- Engel, R. E., Towey, B. D., & Gravens, E. (2015). Degradation of the urease inhibitor NBPT as affected by soil pH. *Soil Science Society of America Journal*, 79(6), 1674-1683.
- Fan, X., Yin, C., Yan, G., Cui, P., Shen, Q., Wang, Q., ... & Liang, Y. (2018). The contrasting effects of N-(n-butyl) thiophosphoric triamide (NBPT) on N₂O emissions in arable soils differing in pH are underlain by complex microbial mechanisms. *Science of the total environment*, 642, 155-167.
- Feng, J., Zhu, Y., Shentu, J., Lu, Z., He, Y., & Xu, J. (2020). Pollution adaptive responses of root-associated microbiomes induced the promoted but different attenuation of soil residual lindane: Differences between maize and soybean. *Science of the Total Environment*, 732, 139170.
- Fierro-Coronado, R. A., Quiroz-Figueroa, F. R., García-Pérez, L. M., Ramírez-Chávez, E., Molina-Torres, J., & Maldonado-Mendoza, I. E. (2014). IAA-producing rhizobacteria from chickpea (*Cicer arietinum* L.) induce changes in root architecture and increase root biomass. *Canadian journal of microbiology*, 60(10), 639-648.
- Fox, T. R. (2004). Nitrogen mineralization following fertilization of Douglas-fir forests with urea in western Washington. *Soil Science Society of America Journal*, 68(5), 1720-1728.
- Karimi, B., Dequiedt, S., Terrat, S., Jolivet, C., Arrouays, D., Wincker, P., ... Ranjard, L. (2019). Biogeography of Soil Bacterial Networks along a Gradient of Cropping Intensity. *Scientific Reports*, 9(1).
- Lee, T. H., Khan, Z., Kim, S. Y., & Lee, K. R. (2019). (thio)hydantoin and hydantoin derivatives from the roots of *Armoracia rusticana* and their neurotrophic and anti-neuroinflammatory activities. *Journal of natural products*, 82(11), 3020-3024.
- Li, W., Xiao, Q., Hu, C., Liu, B., & Sun, R. (2019). A comparison of the efficiency of

- different urease inhibitors and their effects on soil prokaryotic community in a short-term incubation experiment. *Geoderma*, 354, 113877.
- Li, X., Zhang, Z., Luo, J., Cui, X., Xu, J., Hou, Y., ... & Guo, W. (2022). Arbuscular mycorrhizal fungi and nitrilotriacetic acid regulated *Suaeda salsa* growth in Cd-contaminated saline soil by driving rhizosphere bacterial assemblages. *Environmental and Experimental Botany*, 193, 104669.
- Liu, C., Zhang, Y., Liu, H., Liu, X., Ren, D., Wang, L., ... & Zhang, M. (2022). Fertilizer stabilizers reduce nitrous oxide emissions from agricultural soil by targeting microbial nitrogen transformations. *Science of The Total Environment*, 806, 151225.
- Lotia, S., Montojo, J., Dong, Y., Bader, G. D., and Pico, A. R. (2013). Cytoscape app store. *Bioinformatics* 29, 1350–1351.
- Gao, X., Li, C., Zhang, M., Wang, R., & Chen, B. (2015). Controlled release urea improved the nitrogen use efficiency, yield and quality of potato (*Solanum tuberosum* L.) on silt loamy soil. *Field crops research*, 181, 60-68.
- Ghodrat, V., Roustaei, M. J., Tadaion, M. S., & Karampour, A. (2012). Yield and yield components of corn (*Zea mays* L.) in response to foliar application with indole butyric acid and gibberellic acid. *American-Eurasian Journal of Agricultural & Environmental Sciences*, 12(9), 1246-1251.
- Gholivand, K., Pooyan, M., Mohammadpanah, F., Pirastefar, F., Junk, P. C., Wang, J., ... & Mani-Varnosfaderani, A. (2019). Synthesis, crystal structure and biological evaluation of new phosphoramidate derivatives as urease inhibitors using docking, QSAR and kinetic studies. *Bioorganic chemistry*, 86, 482-493.
- Majumdar, P., Bathula, C., Basu, S. M., Das, S. K., Agarwal, R., Hati, S., ... & Das, B. B. (2015). Design, synthesis and evaluation of (thio)hydantoin derivatives as potent topoisomerase I (Top1) inhibitors with anticancer activity. *European Journal of Medicinal Chemistry*, 102, 540-551.
- Matilla, M. A., Daddaoua, A., Chini, A., Morel, B., & Krell, T. (2018). An auxin controls bacterial antibiotics production. *Nucleic acids research*, 46(21), 11229-11238.
- Mendes, P., Garbeva, J.M., Raaijmakers. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiol. Rev.*, 37, pp. 634-663.
- Mohite, B. 2013. Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *J. Soil Sci. Plant Nutr.*

- 13(3), 638-649.
- Olesen JM, Bascompte J, Dupont YL, and Jordano P. 2007. The modularity of pollination networks. *Proc. Natl. Acad. Sci. U. S. A.* 104:19891–19896.
- PARKS, D. H.; TYSON, G. W.; HUGENHOLTZ, P.; BEIKO, R. G. STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics*, v. 30, n. 21, p. 3123–3124, 2014.
- Peters, N., & Thiele-Bruhn, S. (2022). Major metabolites of NBPT degradation pathways contribute to urease inhibition in soil. *Chemosphere*, 135163.
- Pii et al., , Y. Pii, T. Mimmo, N. Tomasi, R. Terzano, S. Cesco, C. Crecchio (2015) Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting Rhizobacteria on nutrient acquisition process. A review. *Biol. Fertil. Soils*, 51, pp. 403-415.
- Sh Sadak, M., Abdelhamid, M. T., & Schmidhalter, U. (2015). Effect of foliar application of amino acids on plant yield and some physiological parameters in bean plants irrigated with seawater. *Acta biológica colombiana*, 20(1), 141-152.
- Sasirekha, B., Shivakumar, S., Sullia, S.B. (2012). Statistical optimization for improved indole-3-acetic acid (IAA) production by *Pseudomonas aeruginosa* and demonstration of enhanced plant growth promotion. *J. Soil Sci. Plant Nutr.* 12(4), 863-873.
- Szajdak, L., & Maryganova, V. (2007). Occurrence of IAA auxin in some organic soils. *Agron Res*, 5(2), 175-187.
- Tavares, M. C., Oliveira, K. A., de Fátima, Â., Coltro, W. K., & Santos, J. C. C. (2021). based analytical device with colorimetric detection for urease activity determination in soils and evaluation of potential inhibitors. *Talanta*, 230, 122301.
- Islam, M., Khan, A., Shehzad, M. T., Hameed, A., Ahmed, N., Halim, S. A., ... & Al-Harrasi, A. (2019). Synthesis and characterization of new thiosemicarbazones, as potent urease inhibitors: In vitro and in silico studies. *Bioorganic chemistry*, 87, 155-162.
- Yadav, A. N., Kour, D., Kaur, T., Devi, R., Yadav, A., Dikilitas, M., ... & Saxena, A. K. (2021). Biodiversity, and biotechnological contribution of beneficial soil microbiomes for nutrient cycling, plant growth improvement and nutrient uptake. *Biocatalysis and Agricultural Biotechnology*, 33, 102009.
- Pérez-Izquierdo, L., Zabal-Aguirre, M., González-Martínez, S. C., Buée, M., Verdú, M., Rincón, A., & Goberna, M. (2019). Plant intraspecific variation modulates nutrient

- cycling through its below ground rhizospheric microbiome. *Journal of Ecology*, 107(4), 1594-1605.
- Ullah, I., Dawar, K., Tariq, M., Sharif, M., Fahad, S., Adnan, M., ... & Arif, M. (2022). Gibberellic acid and urease inhibitor optimize nitrogen uptake and yield of maize at varying nitrogen levels under changing climate. *Environmental Science and Pollution Research*, 29(5), 6568-6577.
- Velasco-Jiménez, A., Castellanos-Hernández, O., Acevedo-Hernández, G., Aarland, R. C., & Rodríguez-Sahagún, A. (2020). Bacterias rizosféricas con beneficios potenciales en la agricultura. *Terra Latinoamericana*, 38(2), 333-345.
- Watts, D.J., Strogatz, S.H. (1998) Collective dynamics of 'small-world' networks. *Nature*, 393, 440-442.
- Wang, S., Wang, X., Han, X., & Deng, Y. (2018). Higher precipitation strengthens the microbial interactions in semi-arid grassland soils. *Global Ecology and Biogeography*, 27(5), 570–580.
- Zhang, G., & Sun, H. J. (2014). Racemization in reverse: evidence that D-amino acid toxicity on Earth is controlled by bacteria with racemases. *Plos one*, 9(3), e92101.
- Zhou, J., Deng, Y., Luo, F., He, .Z, Tu, Q., Zhi, X. (2010). Functional molecular ecological networks. *mBio* 1:e00169-10.
- Zhou, J., Deng, Y., Luo, F., He, Z., & Yang, Y. (2011). Phylogenetic molecular ecological network of soil microbial communities in response to elevated CO₂. *MBio*, 2(4), e00122-11.
- Zobiole, L. H. S., de Oliveira Junior, R. S., Constantin, J., Kremer, R. J., & Biffe, D. F. (2012). Amino acid application can be an alternative to prevent glyphosate injury in glyphosate-resistant soybeans. *Journal of plant nutrition*, 35(2), 268-287.

Supplementary material

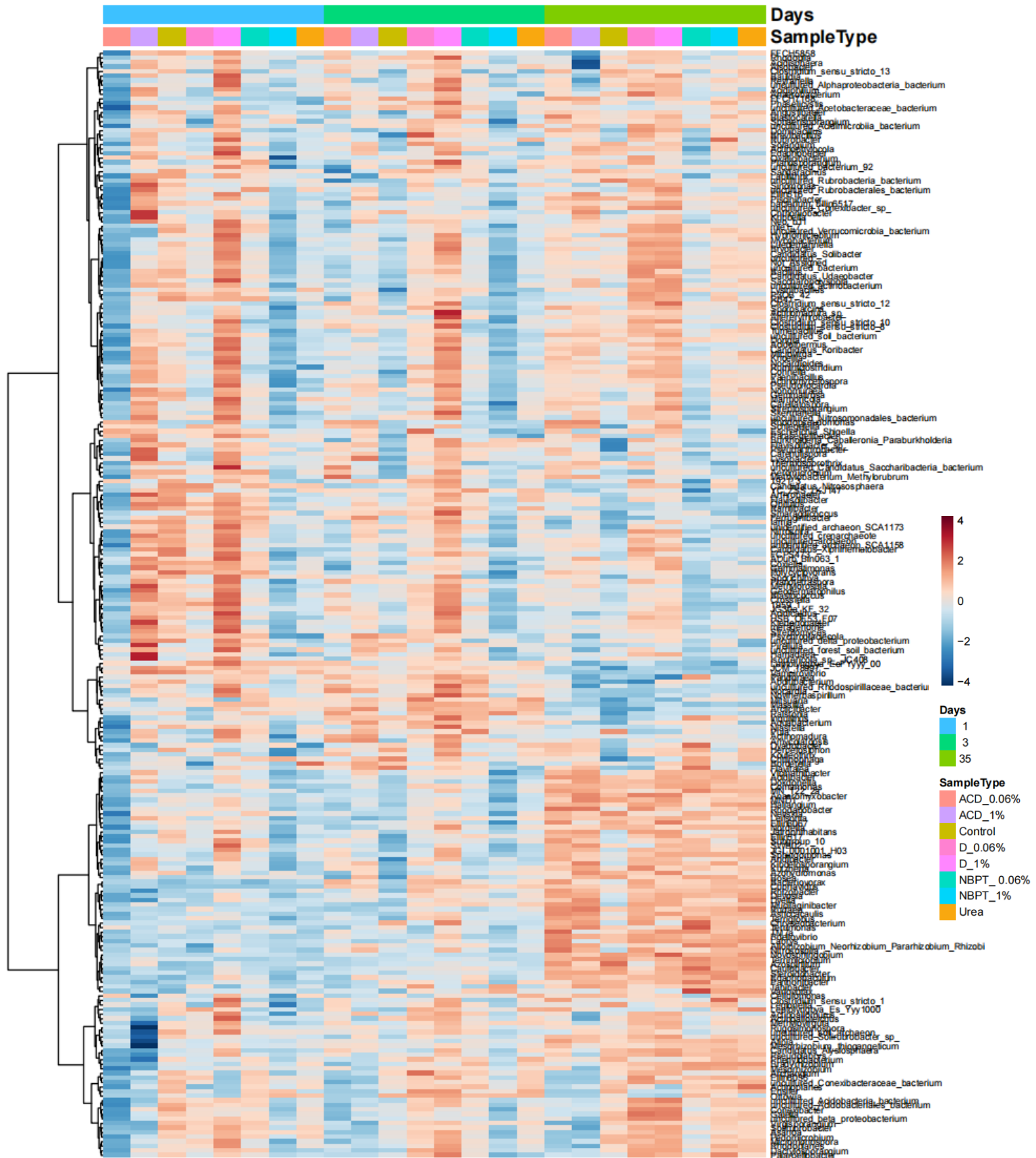


Figure S1. Heatmap with relative abundance of genus.

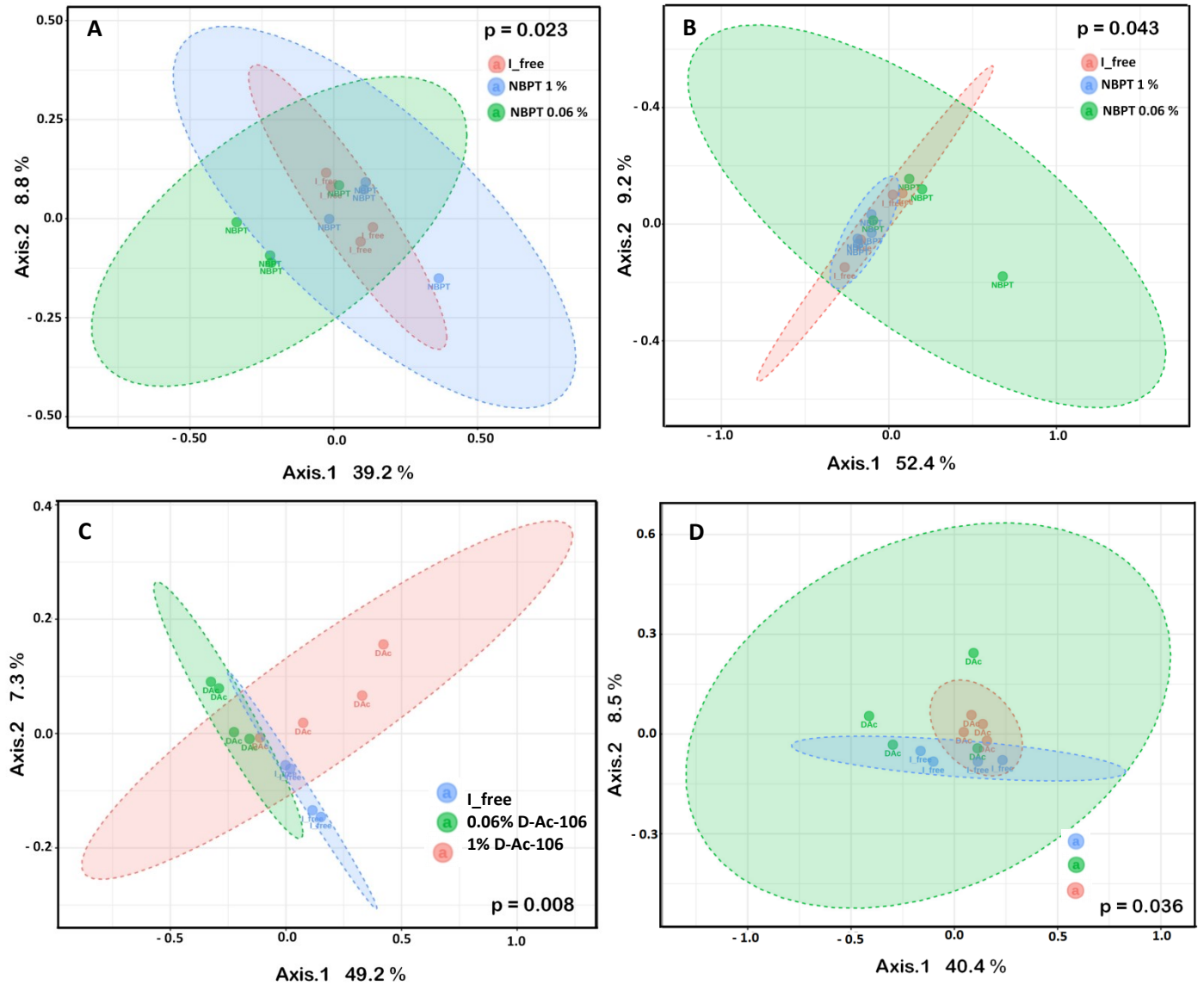
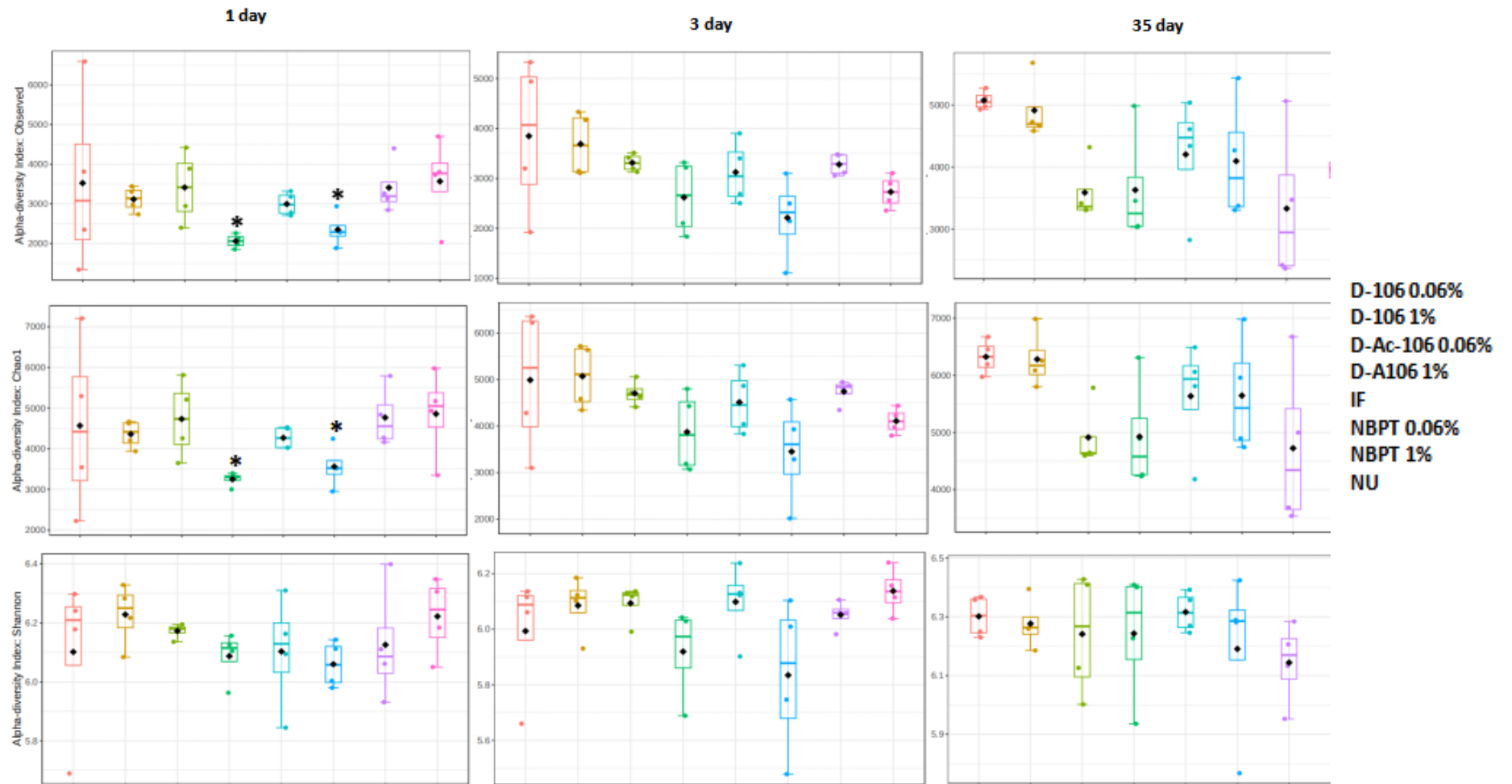


Figure S2. PCOa showing the difference in **A**- microbiome composition on the 1st day after NBPT treatment and **B** - on the 3rd day. **C** - on the 1st day after treatment with D-Ac and **D**- on the 3rd day.



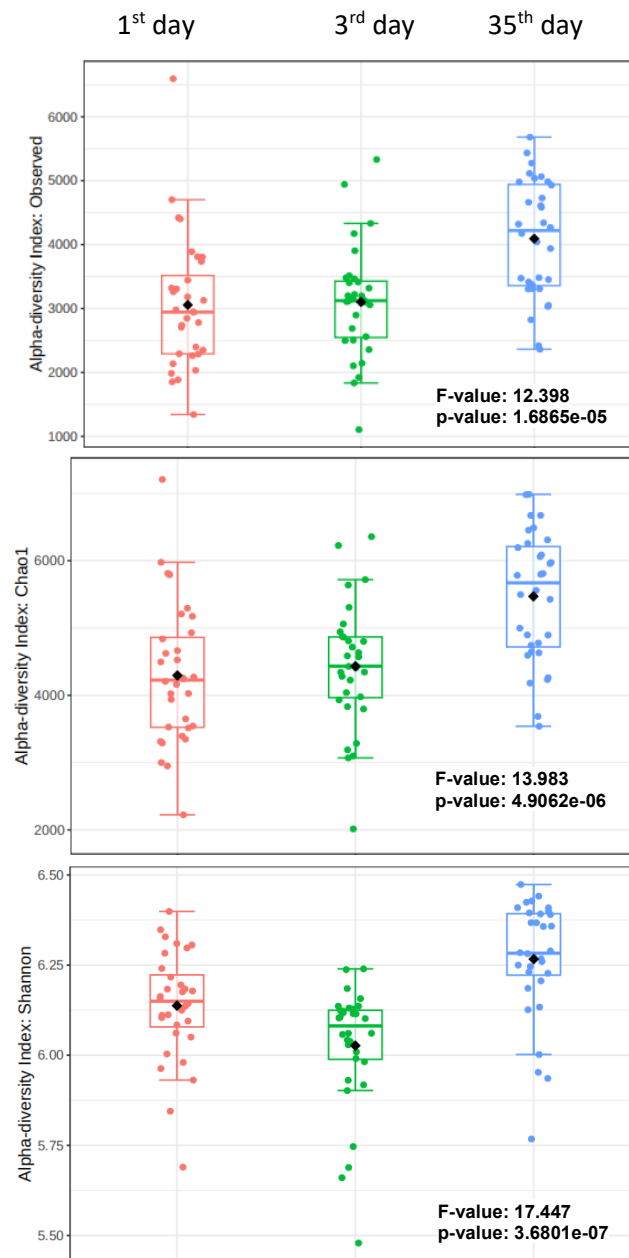


Figure S4. Alpha diversity indices in pearl millet rhizospheric microbiome throughout of experiment.

Table S1. differential abundance of genus treated with inhibitors versus treatment with urea alone. In blue, the genus increased by the presence of inhibitors and in red, the reduced genus.

1 st day				
D-Ac-106 0.06%				
Genus	log2FC	logCPM	Pvalues	FDR
g_Ellin6055	45.957	93.055	1,51E-11	8,81E-09
g_uncultured_actinobacterium	1.658	11.102	3,25E-03	9,01E-01
g_Niastella	17.699	13.391	4,62E-03	9,01E-01
g_Flavisolibacter	1.492	12.519	8,84E-03	0.00012923
g_Conexibacter	13.263	14.244	2.62e-06	0.00030655
g_Sphingomonas	14.817	16.568	3,81E-02	0.00033362
g_Nocardia	14.843	12.003	3,99E-03	0.00033362
g_Kribbella	14.935	11.753	5,26E-02	0.00034725
g_Opitutus	16.475	10.48	5,34E-02	0.00034725
g_Gaiella	13.405	13.676	6,53E-02	0.00038174
g_Blastococcus	13.217	11.927	1,04E-01	0.00051771
g_uncultured_Conexibacter_sp_	14.922	10.898	1,06E-02	0.00051771
g_Caenimonas	13.962	12.418	1,54E-01	0.0006626
g_Pseudolabrys	16.505	11.08	1,59E-01	0.0006626
g_Mesorhizobium	15.508	14.288	2,00E-01	0.00077819
g_Pedomicrobium	14.223	12.574	2,33E-01	0.0008537
g_Unclassified	12.008	17.713	2,63E-01	0.00090495
g_Amycolatopsis	16.719	11.973	2,81E-01	0.00090687
g_MND1	12.465	11.875	3,07E-01	0.00090687
g_uncultured_Solirubrobacter_sp_	14.303	11.184	3,10E-01	0.00090687
g_Streptomyces	12.843	13.636	3,61E-01	0.0010046
g_Solirubrobacter	13.306	14.054	4,65E-01	0.0012362
g_Labrys	15.562	98.635	4,98E-01	0.0012665
g_Bradyrhizobium	11.771	14.875	6,12E-01	0.00139
g_uncultured_bacterium	12.197	16.53	6,16E-01	0.00139
g_Catenulispora	19.565	8.87	6,18E-01	0.00139
g_Rhizobacter	19.679	92.074	7,13E-01	0.0015375
g_Jatrophihabitans	12.178	12.084	7,36E-01	0.0015375
g_Mycobacterium	12.561	12.171	8,32E-01	0.0016774
g_metagenome	13.567	11.889	8,89E-01	0.001734
g_Iamia	15.312	10.302	9,50E-01	0.0017895
g_Ellin6067	11.661	12.265	9,81E-01	0.0017895
g_Reyranella	13.184	12.355	0.00010095	0.0017895
g_Inquilinus	18.618	11.155	0.00010455	0.0017989
g_uncultured_Acidobacteria_bacterium	11.375	13.077	0.00012462	0.0020724
g_Dongia	13.056	12.047	0.00012753	0.0020724
g_Lapillicoccus	11.744	12.032	0.00013265	0.0020973
g_bacterium_Ellin6517	16.446	93.929	0.00013745	0.002116
g_uncultured	11.184	17.208	0.00018244	0.0027366
g_Nordella	11.638	11.468	0.00022212	0.0032486
g_unidentified_archaeon_SCA1158	17.164	90.308	0.00027052	0.003687
g_Kitasatospora	12.959	11.122	0.00027895	0.003687
g_Arthrobacter	12.294	11.743	0.00028106	0.003687
g_Nocardioides	11.787	12.786	0.00028543	0.003687
g_Acidothermus	10.597	12.728	0.00028551	0.003687
g_Geodermatophilus	14.646	9.775	0.00028992	0.003687
g_Devesia	12.577	13.295	0.00029802	0.003691
g_Bacillus	11.421	14.638	0.00030286	0.003691
g_Anaeromyxobacter	11.087	11.809	0.00038575	0.0046054
g_Steroidobacter	11.168	11.781	0.00039889	0.004667
g_Ellin516	16.144	90.868	0.00047126	0.0052581
g_Bryobacter	11.034	12.965	0.00047225	0.0052581
g_Paenibacillus	11.213	12.347	0.00047637	0.0052581
g_Phenylobacterium	1.194	11.382	0.00056669	0.0061392
g_Neo_b11	1.235	10.389	0.00067161	0.0070374
g_Puia	15.825	10.888	0.00067367	0.0070374

g_Rubrobacter	11.955	11.067	0.0007417	0.0076122
g_Candidatus_Udaeobacter	10.787	13.85	0.0011085	0.011181
g_Vampirovibrio	12.252	10.549	0.0011529	0.011431
g_Dyella	1.442	10.082	0.0012103	0.011757
g_Crossiella	11.871	10.925	0.001226	0.011757
g_Haliangium	11.814	11.291	0.001254	0.011832
g_Rhodoplanes	11.857	11.946	0.0013575	0.012478
g_1959_1	13.066	10.594	0.0013652	0.012478
g_Asticcacaulis	15.088	10.855	0.0014367	0.012795
g_Sinomonas	14.109	89.587	0.0014435	0.012795
g_Luedemannella	11.014	11.498	0.0015353	0.013406
g_Dokdonella	15.889	88.989	0.0016636	0.014312
g_uncultured_Alphaproteobacteria_bacterium	11.117	10.681	0.0016994	0.014408
g_Alsobacter	14.276	10.061	0.0021002	0.017551
g_P3OB_42	1.205	10.221	0.0022404	0.01846
g_Candidatus_Solibacter	11.582	12.581	0.0023758	0.019303
g_uncultured_Acidobacteriales_bacterium	10.212	12.772	0.0024526	0.019564
g_Methylobacterium_Methylorubrum	13.574	8.868	0.0024748	0.019564
g_Actinomadura_sp_	11.968	10.42	0.0025519	0.019725
g_Gemmatimonas	11.534	11.548	0.0025626	0.019725
g_Mastigocladopsis_PCC_10914	40.658	91.084	0.0028309	0.021507
g_RB41	12.766	12.718	0.0028801	0.021601
g_Ramlibacter	10.379	11.23	0.003051	0.022593
g_uncultured_Verrucomicrobia_bacterium	12.974	98.534	0.0033516	0.024509
g_Allorhizobium_Neorrhizobium_Pararhizobium_Rhizobium	1.393	12.236	0.0035288	0.025486
g_Actinoplanes	10.411	10.64	0.0037289	0.026602
g_Candidatus_Koribacter	12.189	9.271	0.0038379	0.02705
g_Acidisphaera	11.305	98.054	0.0039198	0.027233
g_Chroococcidiopsis_PCC_7203	32.431	84.567	0.003992	0.027233
g_Mucilaginibacter	12.357	11.229	0.0040035	0.027233
g_uncultured_soil_bacterium	11.875	94.875	0.0041537	0.02793
g_Patulibacter	12.406	97.717	0.0042485	0.028243
g_Vulgatibacter	21.106	79.201	0.0043757	0.028762
g_Subgroup_10	18.545	79.144	0.0047457	0.030847
g_uncultured_archaeon	13.482	88.554	0.0049709	0.031956
g_Nannocystis	-31.718	79.759	0.00506	0.032175
g_uncultured_Candidatus_Saccharibacteria_bacterium	17.856	81.208	0.0053121	0.033415
g_Bauldia	14.146	9.067	0.0059631	0.037111
g_Aminobacter	13.775	89.342	0.0060861	0.037478
g_Hyphomicrobium	10.963	10.151	0.0061733	0.037618
g_Scytonema_UCFS19	32.171	82.175	0.0063429	0.038254
g_Microvirga	10.469	95.331	0.0064553	0.038534
g_uncultured_beta_proteobacterium	11.754	10.5	0.0069263	0.040928
g_Nitrospira	0.90513	13.574	0.0079695	0.046621
g_Acidibacter	0.90727	11.188	0.0086087	0.049862
D-Ac-106 1%				
g_Ellin6055	51.357	8.514	2,34E-10	1,37E-07
3st day				
D-Ac-106 0.06%				
g_Aminobacter	35.413	81.362	2,92E-02	0.0017097
D-Ac-106 1%				
g_Aminobacter	35.889	78.426	9,03E-04	5,28E-01
g_Luteolibacter	-27.126	79.331	7,03E-02	0.0020554
g_Amycolatopsis	-19.998	12.896	6,39E-01	0.012455
g_Edaphobaculum	-13.985	99.853	0.00028156	0.041178
g_Chitinophaga	-14.909	15.126	0.00037376	0.04373
D-106 1%				
g_Aminobacter	34.307	77.628	8,21E-03	0.00048022
35st day				
D-Ac-106 0.06%				
g_Acidicardus	59.164	97.543	4,07E-21	2,38E-18

g_Alsobacter	30.867	90.768	1,54E-02	0.00045079
g_wastewater_metagenome	4.673	76.546	3,62E-02	0.00070528
g_Planotetraspora	29.999	63.603	0.00022297	0.03261

D-Ac-106 1%

g_Acidicoccus	68.815	96.538	6,11E-25	3,57E-22
g_Alsobacter	35.435	8.969	6,69E-05	1,96E-02
g_wastewater_metagenome	46.326	75.708	1,54E-02	0.00030013
g_Acidisphaera	22.374	85.161	4,49E-02	0.00065633
g_Planotetraspora	29.298	62.918	7,40E-01	0.008657
g_uncultured_Alphaproteobacteria_bacterium	15.015	98.455	0.00021795	0.02125
g_Armatimonadetes_bacterium_55_13	-18.177	79.787	0.00037738	0.031538
g_Luteimonas	-28.497	74.725	0.00059532	0.043533

D 0.06%

g_wastewater_metagenome	47.938	71.516	1,43E-03	8,35E-01
g_Azospirillum	27.979	91.336	2,00E-01	0.0052455
g_uncultured_Bacteroidetes_bacterium	-18.074	78.094	2.69e-05	0.0052455

D 1%

g_Planotetraspora	35.777	5.718	1,22E-03	7,14E-01
g_wastewater_metagenome	4.187	71.084	1,18E-02	0.00034374
g_Coxiella	-15.709	74.815	0.00030287	0.045084
g_Oryzihumus	37.901	58.144	0.00030827	0.045084

NBPT 0.06%

g_wastewater_metagenome	-39.475	78.442	2,65E-01	0.015518
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NBPT 1%

g_wastewater_metagenome	-47.684	75.121	9,96E-03	0.00058256
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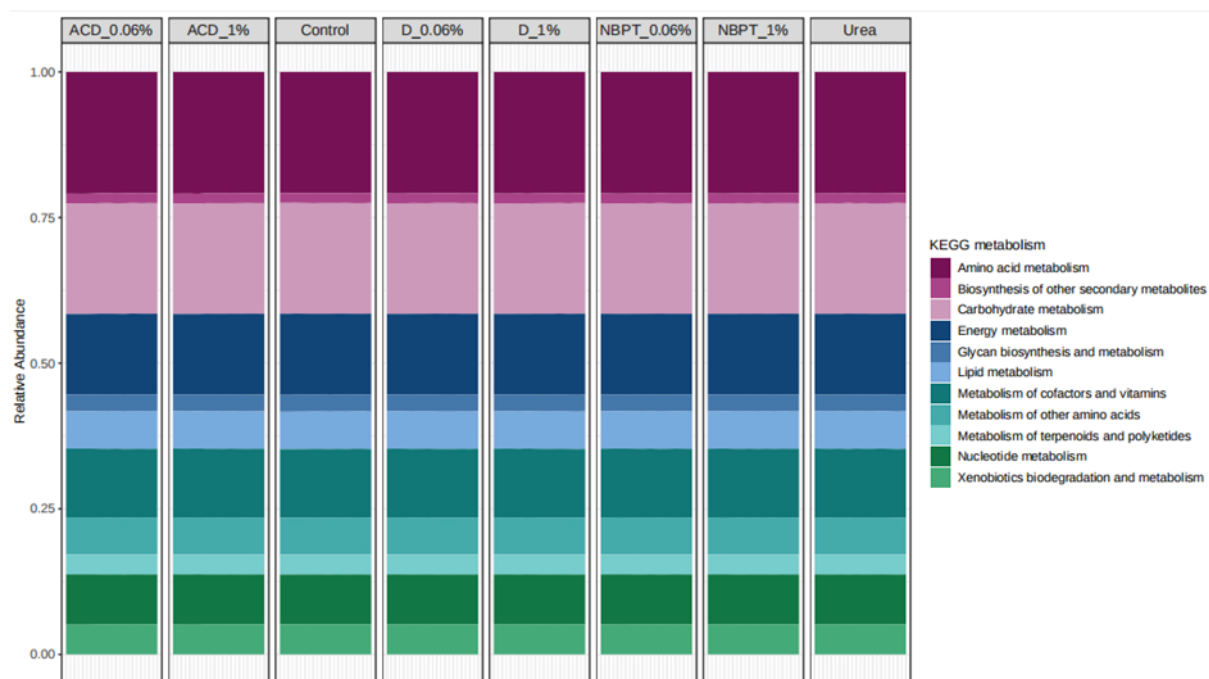


Figure S5. Main metabolic pathways associated with the microbial community in millet rhizosphere.

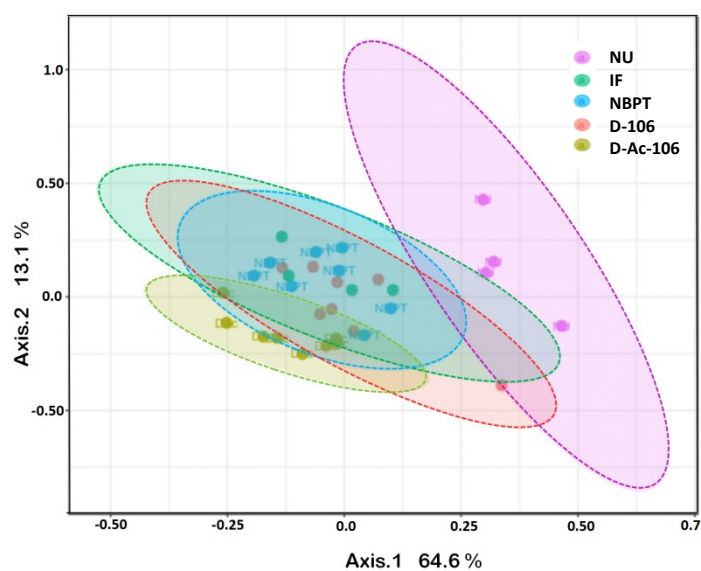


Figure S6. PCoA of functional genes at the end of experiment.

Table S2. Differences on functional prediction between treatment with and without urea on the 3th day after soil fertilization.

Pathway	Size	Hits	Statistic Q	Expected Q	Pval	Holm p
Nicotinate and nicotinamide metabolism	48	24	99.85	14.28	1.09e-15	1.42e-13
Ubiquinone and other terpenoid-quinone biosynthesis	50	21	98.13	14.28	6.38e-13	8.24e-11
Pentose phosphate pathway	67	43	96.68	14.28	5.47e-11	7.00e-09
Methane metabolism	147	78	93.65	14.28	2.83e-10	3.60e-08
Glycine, serine and threonine metabolism	91	64	97.21	14.28	1.13e-09	1.43e-07
Biotin metabolism	19	14	83.16	14.28	3.86e-06	0.00047
Purine metabolism	179	83	72.48	14.28	5.99e-06	0.00074
Carbon metabolism	299	162	71.89	14.28	1.61e-05	0.0019
Isoquinoline alkaloid biosynthesis	34	7	72.75	14.28	4.72e-05	0.0057
Glycerolipid metabolism	61	21	82.32	14.28	5.00e-05	0.0060
Tyrosine metabolism	67	32	74.55	14.28	0.00011	0.013
Phenylalanine metabolism	76	37	74.07	14.28	0.00013	0.016
Tryptophan metabolism	61	26	84.19	14.28	0.00016	0.019
Steroid biosynthesis	28	1	89.33	14.28	0.00039	0.046
Starch and sucrose metabolism	87	49	70.83	14.28	0.00041	0.048

Table S3. Differences on functional prediction between treatment with urea and D-Ac-106 on the 35th day after soil fertilization.

Pathway	Size	Hits	Statistic Q	Expected Q	Pval	Holm p
Xylene degradation	32	16	99.29	9.09	1.48e-23	1.92e-21
Benzoate degradation	82	56	97.12	9.09	1.71e-19	2.21e-17
Pyruvate metabolism	86	60	56.63	9.09	5.23e-09	6.69e-07
Degradation of aromatic compounds	171	84	63.32	9.09	1.81e-07	2.31e-05
Aminobenzoate degradation	35	26	62.35	9.09	1.77e-06	0.00022
Porphyrin and chlorophyll metabolism	76	60	58.83	9.09	4.03e-05	0.0050
Lipopolysaccharide biosynthesis	17	15	62.76	9.09	4.32e-05	0.0053
Carotenoid biosynthesis	28	8	67.13	9.09	7.71e-05	0.0094
Pentose phosphate pathway	67	43	54.26	9.09	0.00011	0.01
Styrene degradation	19	12	67.61	9.09	0.00031	0.038

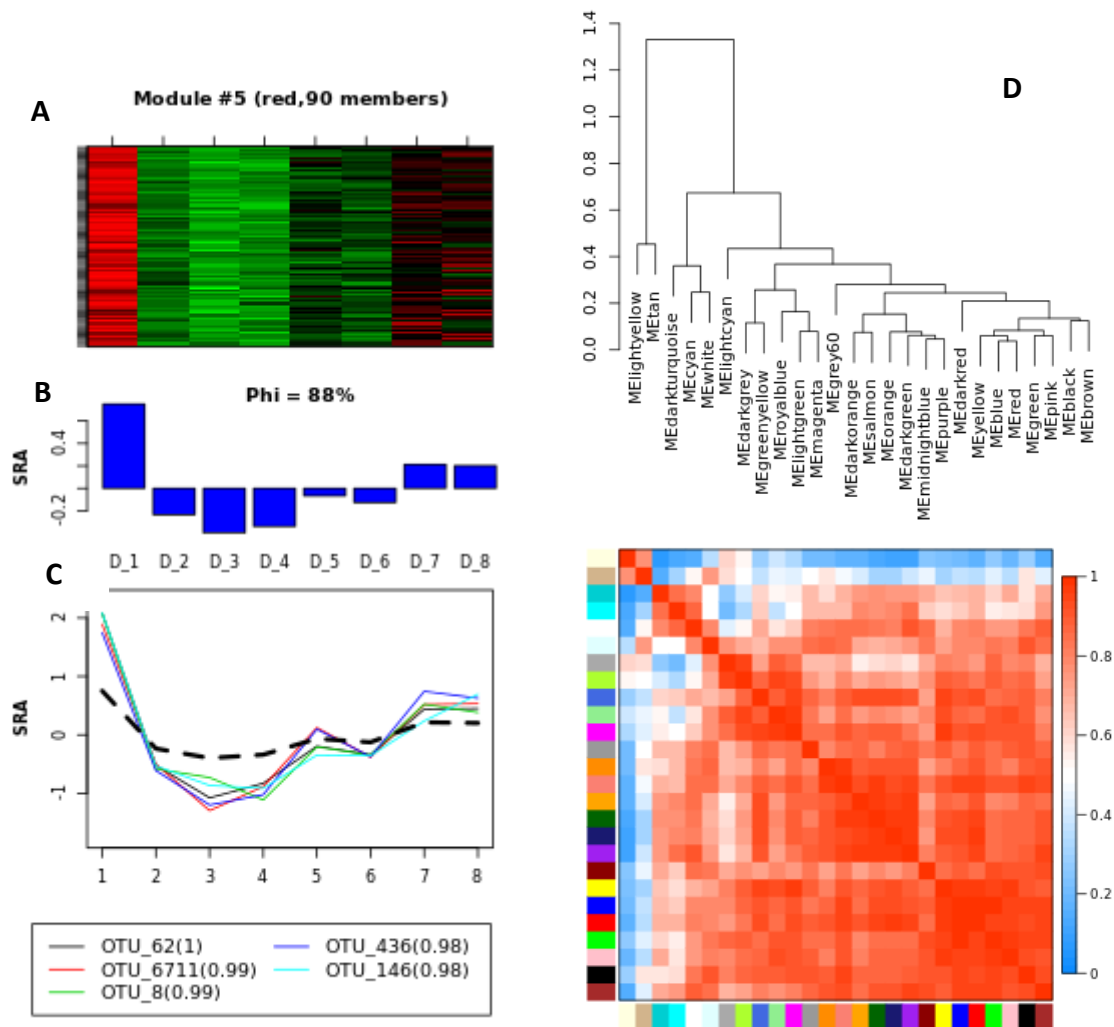


Figure S7. (A) Heat map of the relative abundance (SRA) of OTUs in different samples from the module with the highest number of members after D-106 treatment. Rows correspond to OTUs and columns are samples. Red corresponds to OTUs whose SRAs are >0 and green means those whose SRAs are <0 . (B) SRA of the corresponding autogene (y-axis) in the samples (x-axis). The parameter (Phi) how much the autogene explains the total variance. (C) Relative abundance of groups of OTUs that consistently coexist in these microbial communities. Only 5 significant OTUs are shown, where the y-axis are SRAs and the x-axis are individual samples. The values in parentheses are the module association. (D) The plots show the correlations among modules. Red means a higher correlation, whereas blue means a lower correlation.

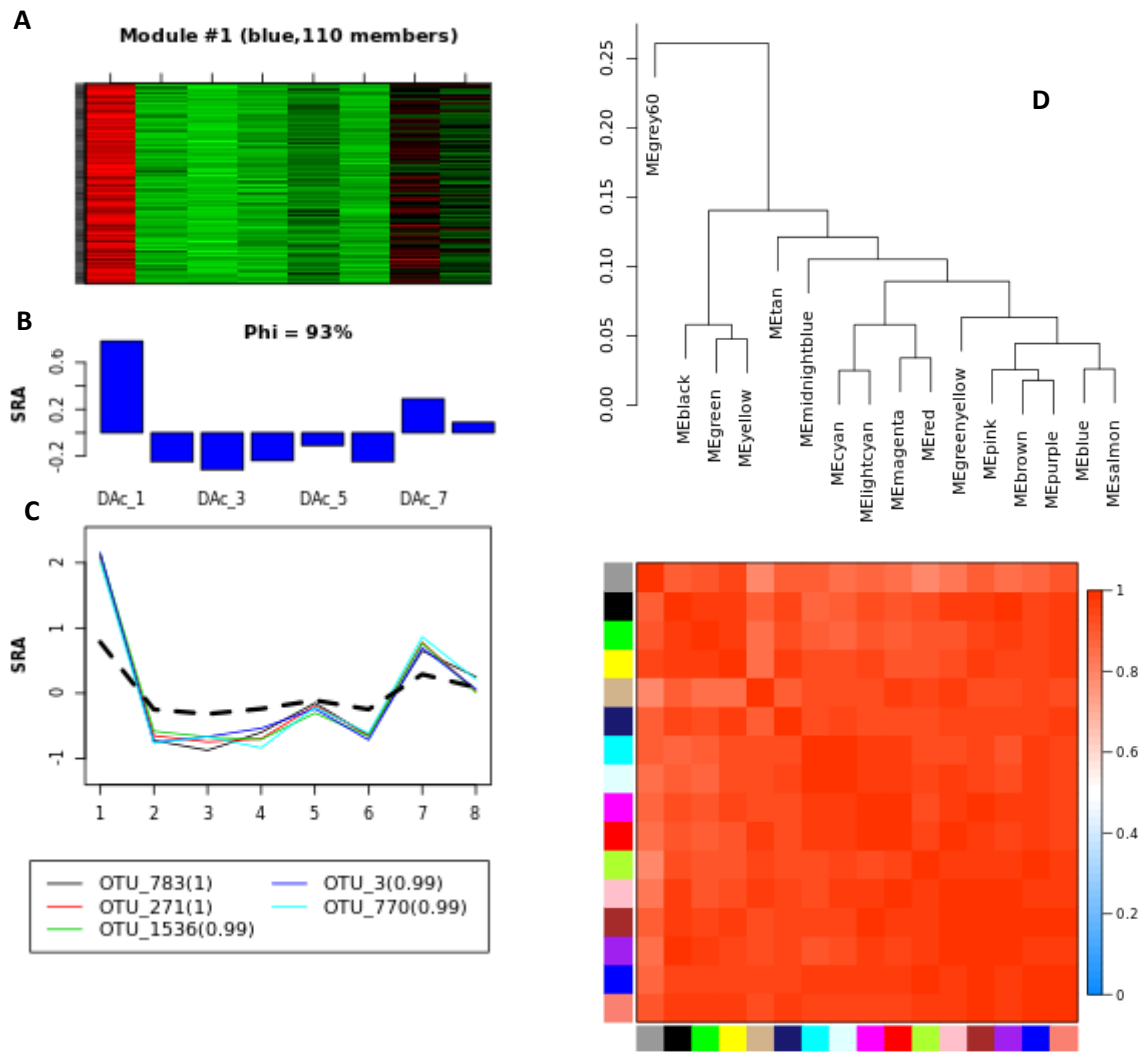


Figure S8. (A) Heat map of the relative abundance (SRA) of OTUs in different samples from the module with the highest number of members after D-Ac-106 treatment. Rows correspond to OTUs and columns are samples. Red corresponds to OTUs whose SRAs are >0 and green means those whose SRAs are <0. (B) SRA of the corresponding autogene (y-axis) in the samples (x-axis). The parameter (Phi) how much the autogene explains the total variance. (C) Relative abundance of groups of OTUs that consistently coexist in these microbial communities. Only 5 significant OTUs are shown, where the y-axis are SRAs and the x-axis are individual samples. The values in parentheses are the module association. (D) The plots show the correlations among modules. Red means a higher correlation, whereas blue means a lower correlation.

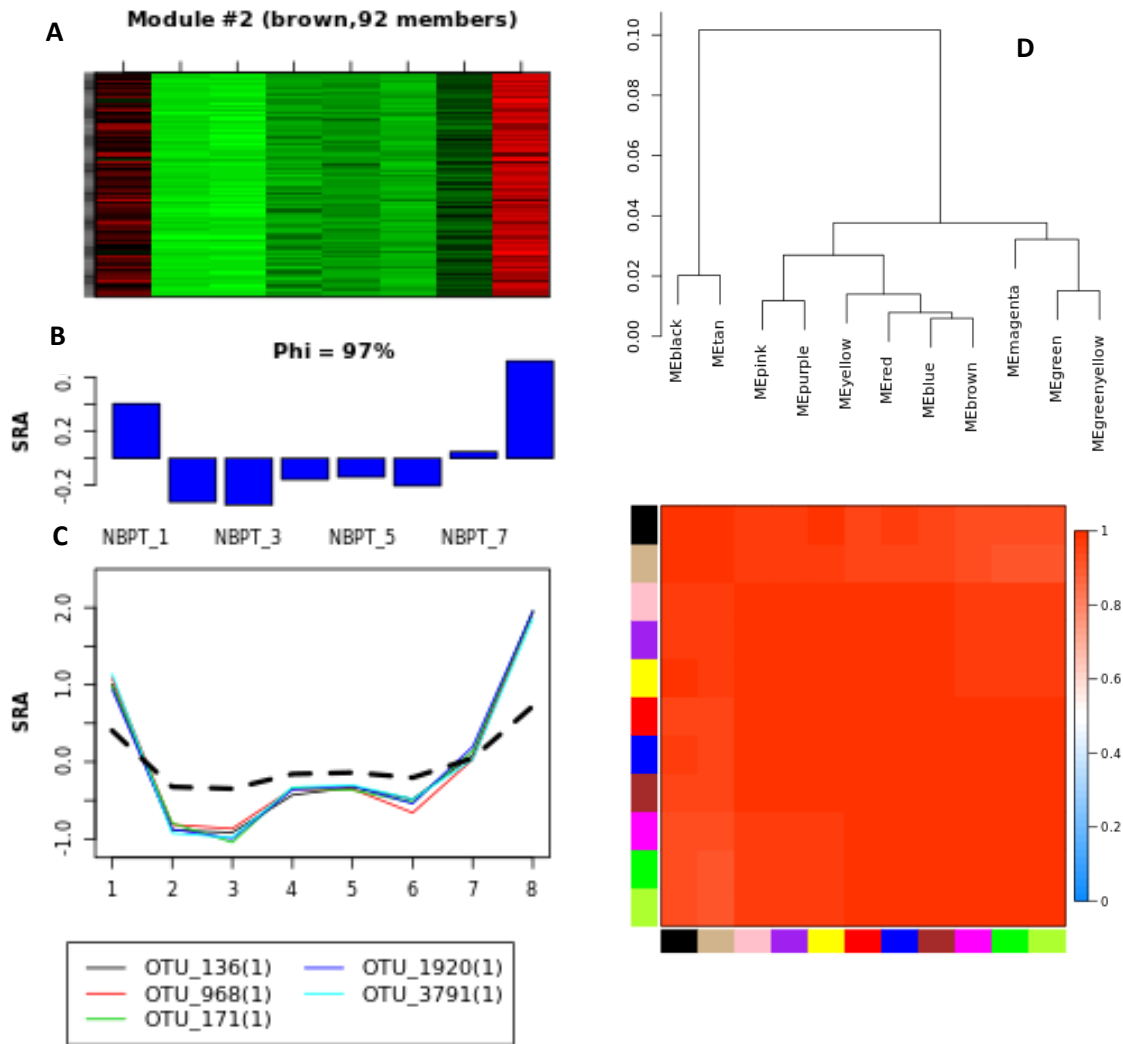


Figure S9. (A) Heat map of the relative abundance (SRA) of OTUs in different samples from the module with the highest number of members after NBPT treatment. Rows correspond to OTUs and columns are samples. Red corresponds to OTUs whose SRAs are >0 and green means those whose SRAs are <0. (B) SRA of the corresponding autogene (y-axis) in the samples (x-axis). The parameter (Phi) how much the autogene explains the total variance. (C) Relative abundance of groups of OTUs that consistently coexist in these microbial communities. Only 5 significant OTUs are shown, where the y-axis are SRAs and the x-axis are individual samples. The values in parentheses are the module association. (D) The plots show the correlations among modules. Red means a higher correlation, whereas blue means a lower correlation.

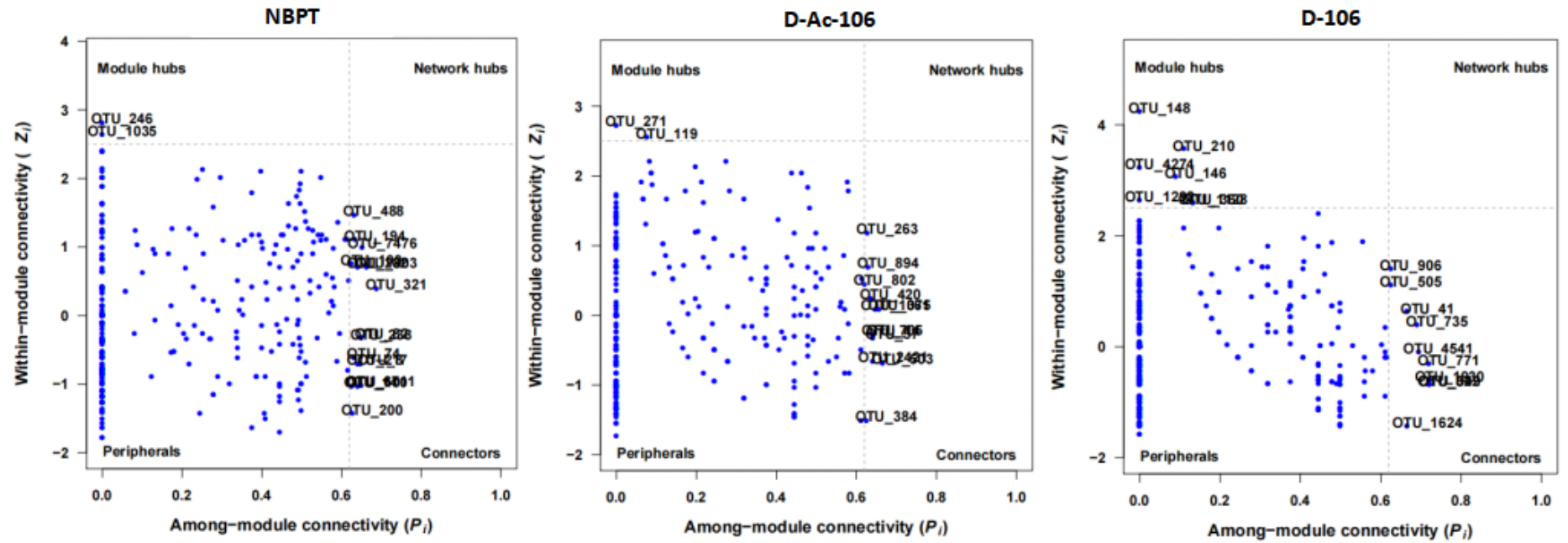


Figure S10. Z-P plot showing distribution of OTUs based on their topological roles. Each symbol represents an OTU. The topological role of each OTU was determined according to the scatter plot of within-module connectivity (Z_i) and among-module connectivity (P_i). The module hubs and connectors are labeled with numbers.

Table S4. Affiliation of keystone OTUs in Z-P graph.

Treatment	Key-microorganisms	Nodes	Taxa
NBPT	Connectos	OTU_74	Gemmatimonadota
		OTU_8	Acidobacteriota
		OTU_238	Acidobacteriota
		OTU_217	Acidobacteriota
		OTU_103	Chloroflexi
		OTU_321	Unclassified
		OTU_200	Patescibacteria
		OTU_82	Actinobacteriota
		OTU_7476	Proteobacteria
		OTU_100	Proteobacteria
		OTU_194	Acidobacteriota
		OTU_6711	Proteobacteria
		OTU_488	Actinobacteriota
		OTU_303	Acidobacteriota
		OTU_14	Actinobacteriota
		OTU_282	Actinobacteriota
	Module Hubs	OTU_246	Actinobacteriota
		OTU_1035	Gemmatimonadota
D-106	Connectos	OTU_41	Actinobacteriota
		OTU_349	Unclassified
		OTU_735	Myxococcota
		OTU_4541	Proteobacteria
		OTU_771	Chloroflexi
		OTU_1624	Actinobacteriota
		OTU_822	Proteobacteria
		OTU_906	Proteobacteria
		OTU_505	Verrucomicrobiota
		OTU_1930	Proteobacteria
	Module Hubs	OTU_210	Actinobacteriota
		OTU_360	Actinobacteriota
		OTU_146	Unclassified
		OTU_148	Proteobacteria
		OTU_1292	Unclassified
		OTU_4274	Proteobacteria
		OTU_1128	Acidobacteriota

D-Ac-106	Connectos	OTU_420	Chloroflexi
		OTU_2421	Chloroflexi
		OTU_503	Chloroflexi
		OTU_263	Proteobacteria
		OTU_57	Actinobacteriota
		OTU_384	Chloroflexi
		OTU_705	Unclassified
		OTU_14	Actinobacteriota
		OTU_1075	Chloroflexi
		OTU_361	Actinobacteriota
		OTU_894	Proteobacteria
	Module Hubs	OTU_271	Actinobacteriota
		OTU_119	Chloroflexi

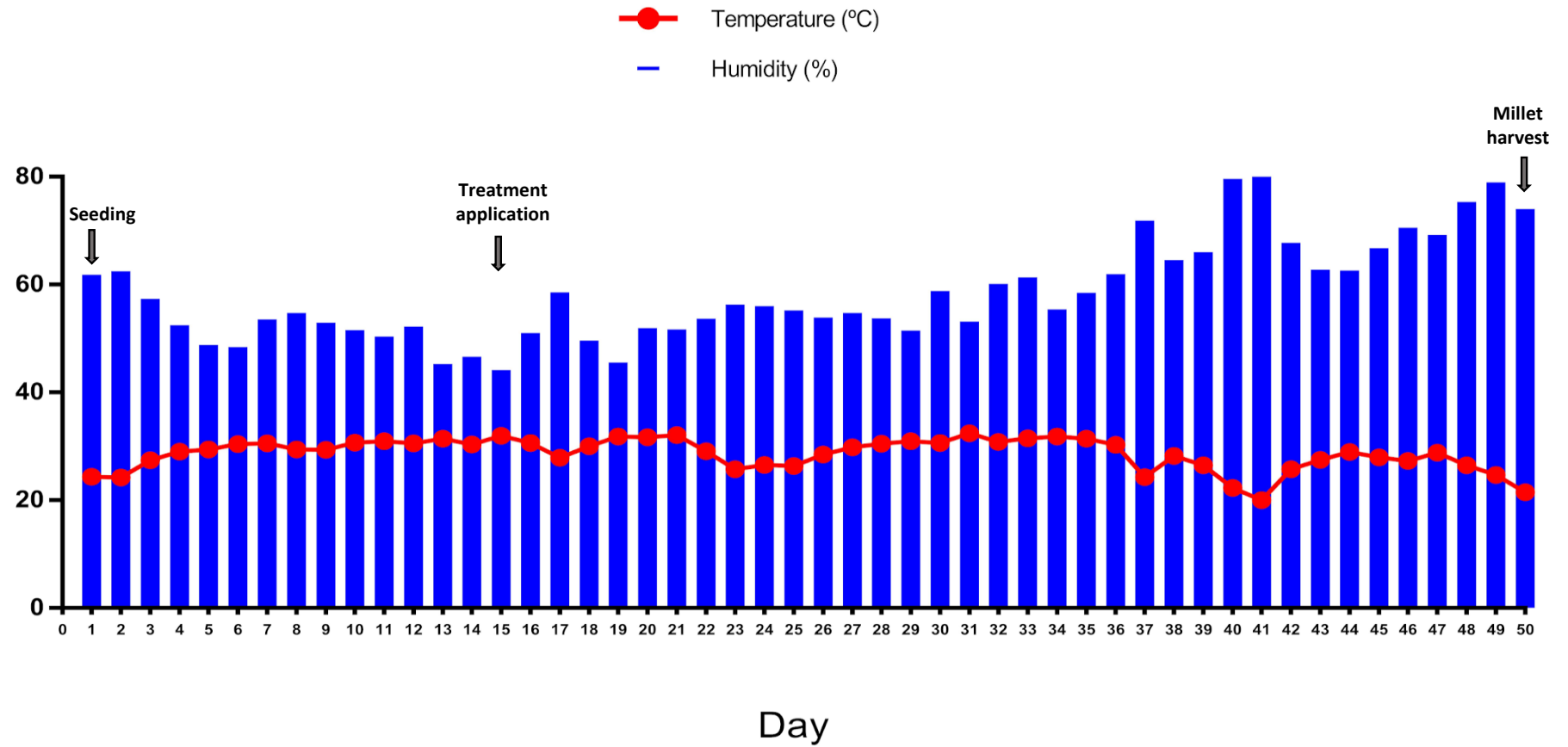


Figure S11. Temperature and humidity throughout the entire experiment carried out in a greenhouse.

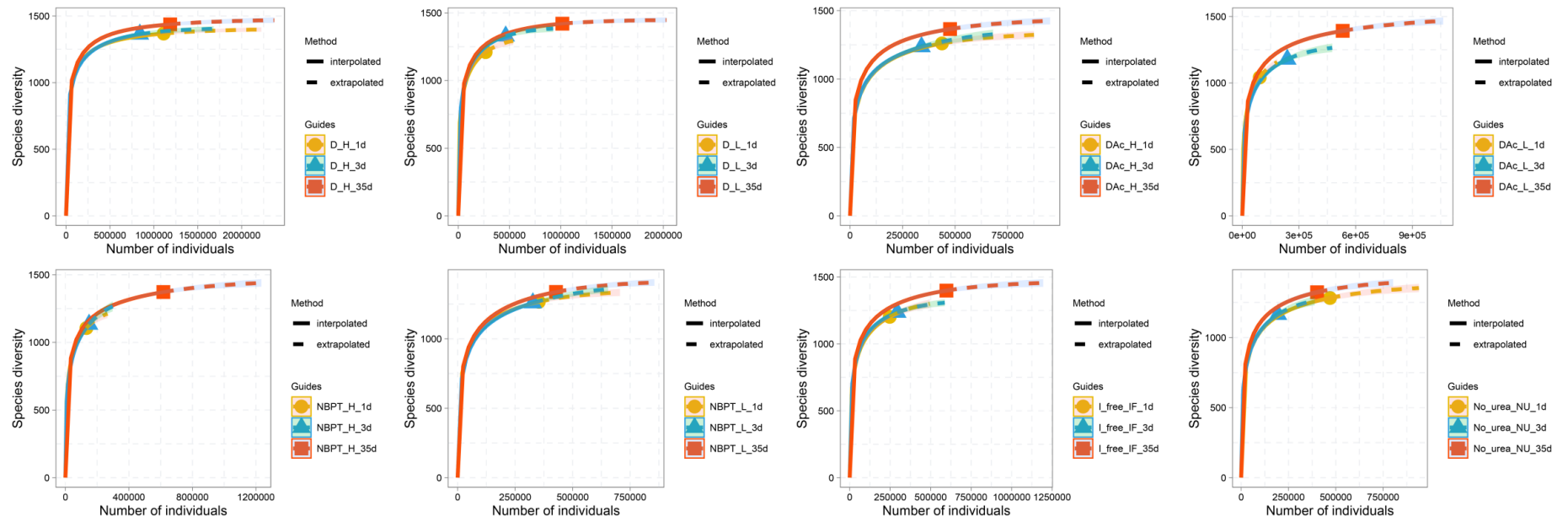


Figure S12. Rarefaction curves

Chapter 5

Naphthoquinone derivatives impair the growth of *Helicobacter pylori* and gastric tumor cells

Abstract

Lapachol and lawsone are naphthoquinones widely found in plants. Despite some reports on the inhibition of urease by lawsone derivatives, little is known about the potential of lapachol and lawsone gluco- and gluco-triazol derivatives on the ureolytic activity of urease, a virulence factor for the pathogen bacteria *Helicobacter pylori*. Some studies have shown the cytotoxic effect of these substances against several cancer strains. Therefore, the present study aimed to evaluate the ability of lapachol and lawsone gluco- and gluco-triazoles derivatives to inhibit the growth of *H. pylori* and stomach cancer cells. The best inhibitors determined from *in vitro* experiments with purified urease from *Canavalia ensiformis* (> 75% enzyme inhibition) were investigated for their type of enzyme inhibition and also selected for the tests with *H. pylori* and cancer gastric cells. Five derivatives (Plaw, Flaw, Law15, Law16, Lap1) out of 31 tested completely inhibited the urease from *C. ensiformis* and showed an inhibition profile typical of mixed inhibitors. Molecular docking using the tridimensional structure of *H. pylori* urease (5LP4) revealed that LAW 15 and LAW 16 destabilize the active site of urease. Naphthoquinone derivatives (PLAU, LAW15, LAW16, LAP1) efficiently inhibited the *H. pylori* growth at minimum inhibitory concentrations (MICs) ranging from 64 to 128 µg/mL. Two compounds (PLAU and FLAU) prevented the growth of gastric tumor cells with IC₅₀ lower than the reference compound (cisplatin, IC₅₀ = 4.5ug/mL). Glycosylated naphthoquinones may become an important alternative tool in the prevention and treatment of *H. pylori* infection and cancer gastric tumor.

Keywords: Naphthoquinone glycosides cytotoxicity, *Handroanthus*, Urease inhibitors, *Helicobacter pylori*, Stomach cancer.

1. Introduction

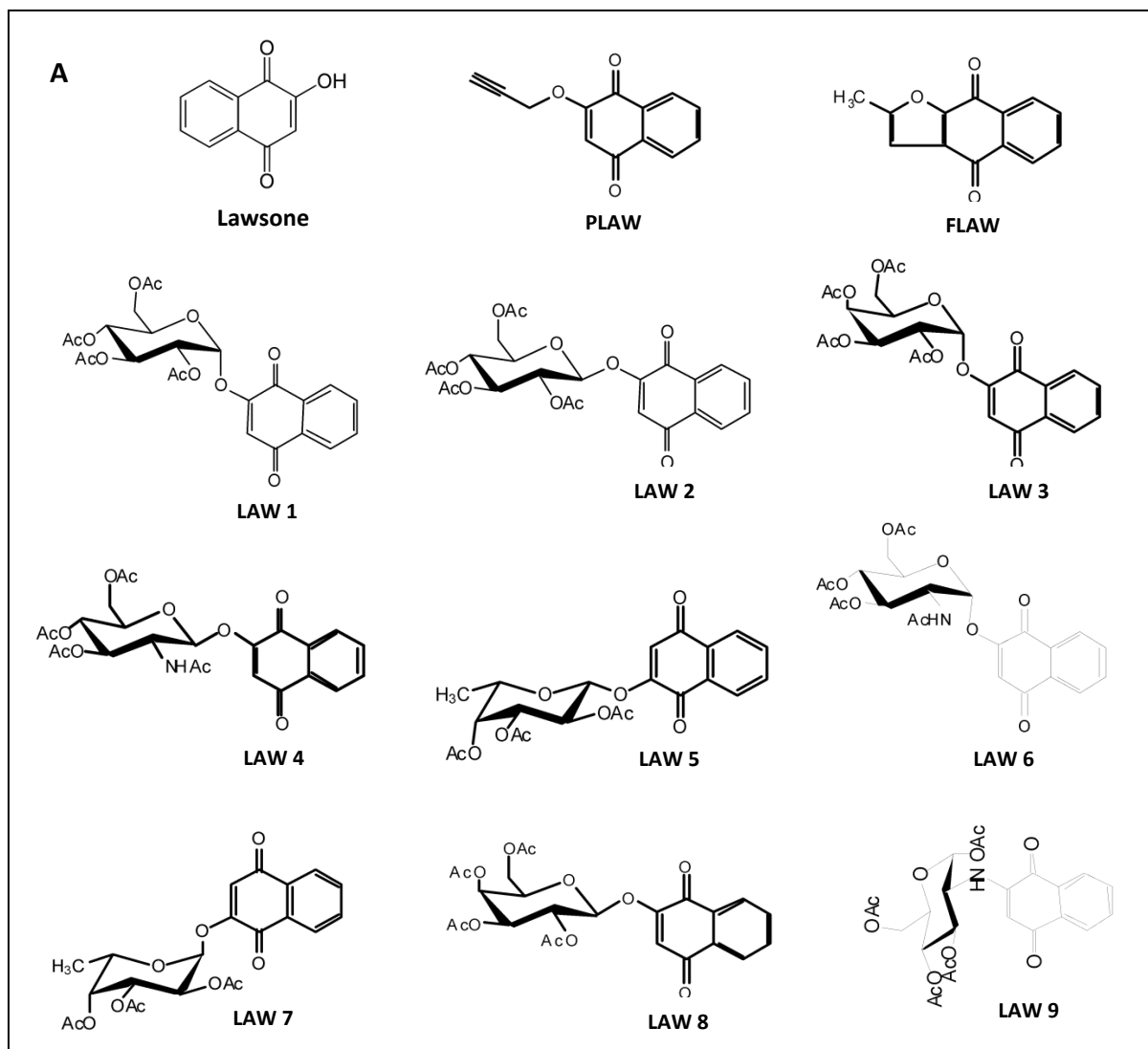
Bacterial resistance to current antibiotics has been of great concern and other options of treatment are being currently investigated to overcome this issue. The resistance of *H. pylori* strains to clarithromycin and metronidazole is a reality that has been alarming in several regions of the world (Alba et al., 2017; Hu et al., 2017). The stomach is the only natural reservoir of *H. pylori* and it is estimated that this bacterium was able to infect over 50% of the world's human population (Alipour, 2021). Such an infection can increase the chance of developing gastric cancer by up to seven times (Wang, 2014), and has been classified as a group I carcinogen by the World Health Organization (WHO) (Crowe, 2019). The development of cancer occurs after bacterial adhesion to the epithelium and the release within cells of the *cagA* protein, which prompts an increase in the levels of cyclins and ultimately uncontrolled cell proliferation (Alipour, 2021). The *cagA*-positive strains are responsible for about 60% of bacterial infections and are implicated in the origin of acute gastritis, peptic ulceration, and gastric cancer (Bourzac and Guillemin, 2005; Takahashi-Kanemitsu et al., 2020).

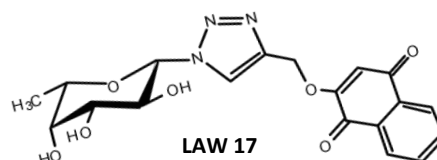
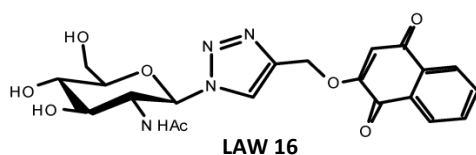
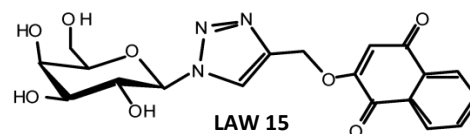
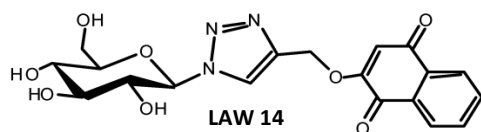
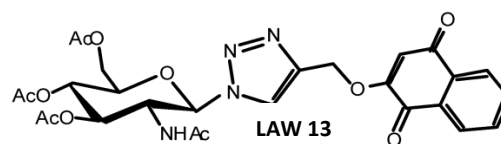
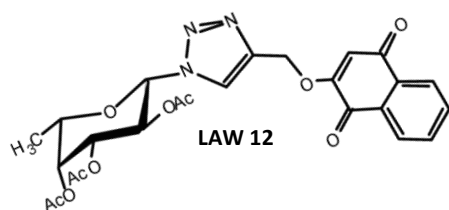
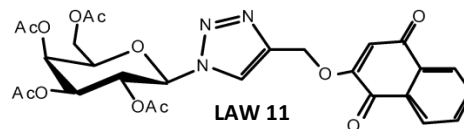
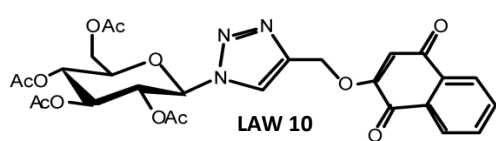
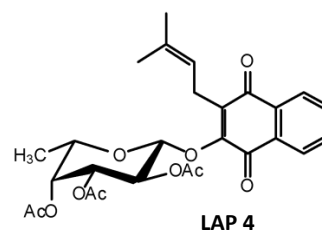
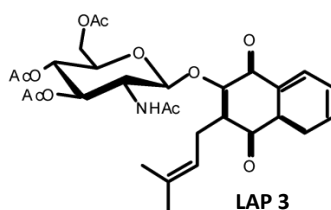
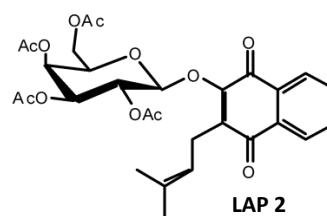
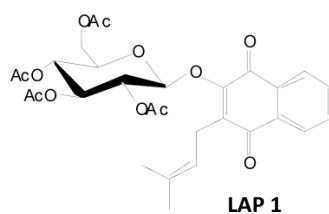
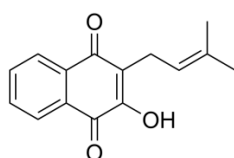
Despite the harsh acidic conditions of the stomach, *H. pylori* is able to penetrate the stomach mucus with the help of flagella (Rush and Engel, 2017). After crossing the mucus, about 20% of *H. pylori* strains are able to bind to the stomach gastric epithelium where they cause chronic inflammation throughout colonization (Fagoonee et al., 2019; Qureshi and Gu, 2019). Urease is a key virulence factor for *H. pylori* that guarantees the successful initial colonization and with the maintenance of the chronic infection due to the urea hydrolysis (Debowski et al., 2017). Urease is a metalloenzyme widely expressed by *H. pylori* capable of converting urea into bicarbonate and ammonia, thus forming a microenvironment with less acidic conditions (Marshall et al., 1990). In addition, ureases also act as adhesins, helping bacteria binding to the gastric epithelium, further causing cell apoptosis (Fan et al., 2000). The death of immune and epithelial cells at the site of infection provides nutrients, which allow the perpetuation of colonization (Takahashi-Kanemitsu et al., 2020).

Thus, urease has been a target, both for the development of an anti-*H. pylori* vaccine (Zeng et al, 2015), and in the search for substances capable of inhibiting and blocking its action (Kafarski and Talma, 2018). Lapachol is a naphthoquinone widely found in plants, such as in the Verbenaceae, Proteaceae and main Bignoniaceae

families (Wenceslau et al., 2006). Lapachol has been extensively studied as an anticancer agent, both for its antiproliferative capacity and antimetastatic effects (Epifanio et al., 2014). However, its limited bioavailability due to poor water solubility provides side effects. Glycosylation of poorly soluble molecules is a powerful tool to increase their solubility and therefore bioavailability.

Lawsonia is a natural bioactive compound present in plants of the *Lawsonia* genus (Ottoni et al., 2020). Plants containing naphthoquinones are widely used in the treatment of tumors and parasitic diseases in Asia and South America (Epifanio et al., 2014). Naphthoquinones have been widely studied as antitumor agents due to their potential to inhibit human DNA topoisomerase II and the production of reactive oxygen species. Considering the state-of-the-art, this work investigated the extent of the inhibition of urease and *H. pylori* and gastric tumor cells (ATCC CRL - 1739) growth by naphthoquinones glucosides derived from lapachol and lawsone .



B**C**

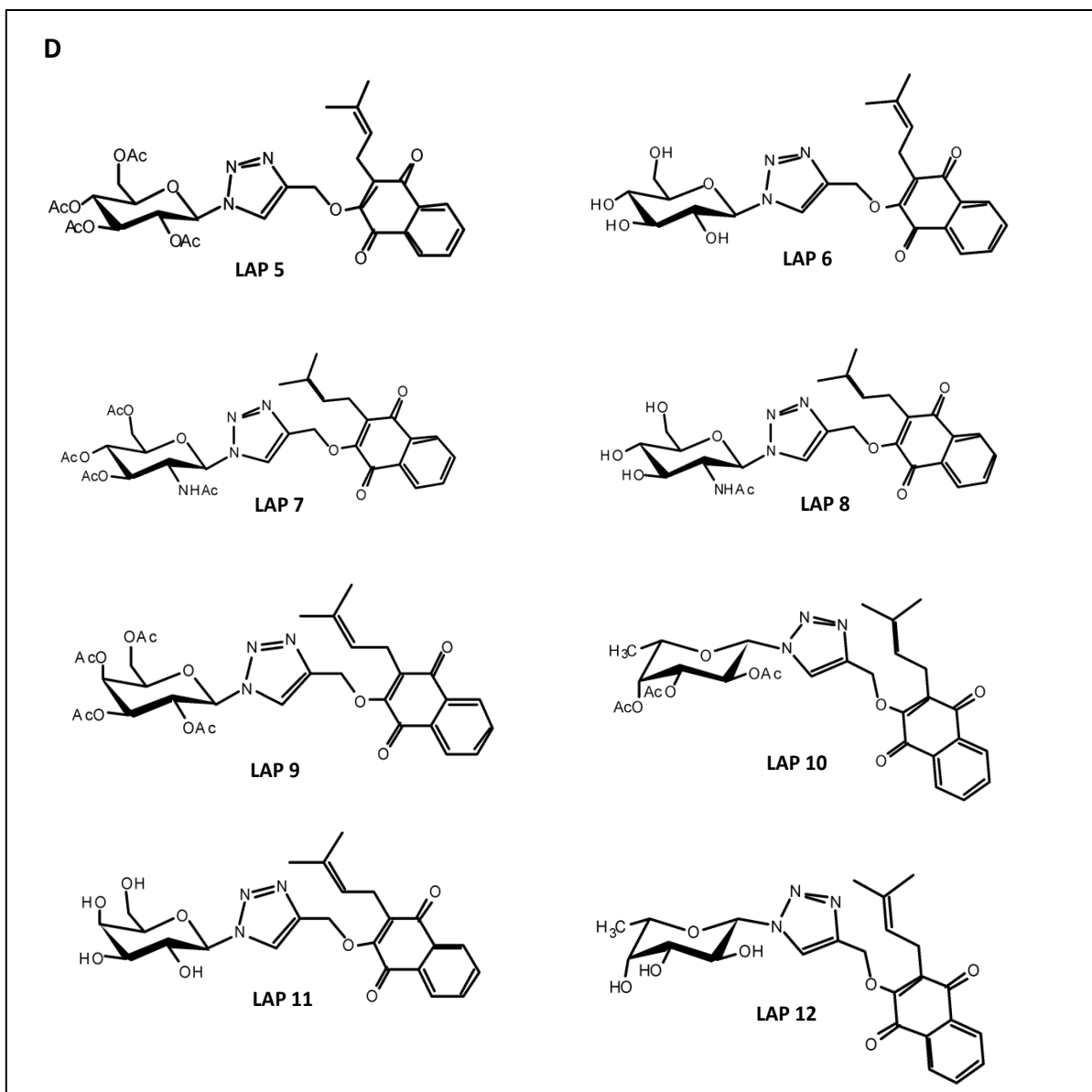


Figure 1 - Lawsone and glycosidic derivatives (**A**), along with a substituted propargyl derivative (PLAW) and methylfuran (FLAW). Derived from the lawsone glycosides triazoles (**B**). Lapachol glycosidic derivatives (**C**) and triazoles glycosides (**D**).

2. Material and Methods

2.1 *In vitro* urease inhibition

The naphthoquinone derivatives were screened for the ability to inhibit *in vitro* the activity of type III urease purified from *Canavalia ensiformis* (jack bean) (Sigma, St. Louis, Mo, USA). For this, urease activity was performed according to (Weatherburn, 1967). The enzyme was incubated in the presence or absence of compounds-test at 500 μ M in a reaction medium containing 20 mM phosphate buffer (pH 7.0), 1 mM ethylene diamine tetraacetic acid (EDTA) and 20 mM urea. The reaction was incubated for 10 min at 25°C and stopped by the addition of a solution containing phenol (1% m/v) and sodium nitroprusside (SNP) (5 mg L⁻¹), followed by the addition of NaOH solution (0.5% m/v) in NaOCl (0.1% v/v). After incubating the samples at 50 °C for 5 min, the absorbance was measured at 630 nm. Hydroxyurea (HU) was used as a reference of urease inhibitor and the percentage of urease inhibition was determined by comparing the NH₄⁺ content formed in the reactions with the test compounds in relation to the control. Three independent experiments were performed, each with four replications.

2.2 Kinetics studies

The inhibition mechanism exhibited by the most potent inhibitors was determined by incubating the inhibitors at concentrations necessary to inhibit jack bean urease activity in the range from 30 to 40% (from 0.01 to 0.16 mM) in reaction containing 20 mM phosphate buffer (pH 7.0), 1 mM EDTA, urea (ranging from 1 to 32 mM) and 12.5 mU urease. Reactions were stopped and the quantification of NH₄⁺ was done as previously described. The Michaelis-Menten hyperbolas and Lineweaver-Burk plots were obtained using the software OriginPro 8 (Origin Lab, Northampton, MA), and equilibrium dissociation constants for the urease inhibitor complex (K_i) and for the urease inhibitor-urea complex (K'_i) were determined using the experimental values of K_M , V_{max} , $K_{M\ app}$, $V_{max\ app}$, α and α' .

2.3 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the tested compounds against *H. pylori*

The MIC and MBC were determined using the broth microdilution method as established by the Clinical & Laboratory Standards Institute (CLSI) (M7-A10, 2015),

with adaptations. In a 96-well microplate was added 100 μL of *H. pylori* suspension (10^6 UFC/mL) in BHI medium and 100 μL of each derivative (32 $\mu\text{g/mL}$ to 1024 $\mu\text{g/mL}$) diluted in BHI medium. The absorbance was recorded at 620 nm and the reactions incubated at 37°C, under 10% CO_2 for 72 h. The reactions were homogenized, and a new reading at 620 nm was performed to determine the MIC values. The concentrations corresponding to the wells in which apparently there was no bacterial growth were defined as the MIC value. MBC was established as the lowest concentration capable of inhibiting colony formation on Columbia Agar Plates containing 5% v/v sheep blood. The tests were performed in independent triplicate, using growth controls (absence of compounds) and negative controls (culture medium containing different concentrations of each compound as a color control). Amoxicillin was used as an antimicrobial standard.

2.4 Molecular docking studies with urease inhibitors and urease from *H. pylori*

The best inhibitors were selected for the study of interaction with the active site of the urease enzyme. Interaction studies were performed with the GOLD software, version 5.5, by fitting calculations into the crystallographic PBP protein complex (PDB code: 5LP4) deposited in the Protein Data Bank (PDB) database, selecting the goldscore scoring function in a sphere radius of 10 Å around the crystallographic ligand. The generated model was validated by redocking and cross docking using the biapenem ligand crystallography. The redocking process consists of removing the crystallographic ligand from the file containing the protein-ligand complex and then reattaching it back to its active site. The deviation from the original bioactive conformation (crystallographic ligand) and the resulting docking pose is then calculated by the root mean squared deviation (RMSD). The cross docking technique will then test the ability of the validated model to solve the calculus in a similar crystallographic complex (of the same protein), verifying its reliability in finding the right solution.

2.5 Evaluation of in vitro cytotoxicity

The Human gastric adenocarcinoma cell line (AGS) (ATCC CRL - 1739) was obtained from the Rio de Janeiro Cell Bank. Cells were maintained in DMEM medium (Dulbecco's modified eagle medium) supplemented with 10% fetal bovine serum (FBS) (v/v) and incubated in an oven at 37°C with an atmosphere containing 5% CO_2 at different periods until they reach confluence of approximately 70-90%. Afterwards, the cells were removed with the aid of a trypsin solution and counted in a Neubauer

chamber to obtain the values of cellular concentration. 200 μ L of DMEM supplemented with approximately 6×10^4 cells/mL (AGS) were distributed in 96-well microplates for culture of tissue and incubated at 37°C, 5% CO₂ for 24 h. After this period, the medium was removed and the adhered cells were treated with 200 μ L of different concentrations of substance-tests in supplemented DMEM and incubated for 48 h at 37°C and 5% CO₂ atmosphere. After incubation, the plates were prepared for the MTT-tetrazolium test (MOSMANN, 1983). Cisplatin was used as the reference of antiproliferative compounds. The tests were accompanied by control growth (without treatment), performed in triplicate and repeated at least 3 times. The tests were carried out from the concentration of 100 μ g/mL for the substances, with at least six concentrations always reducing by a half. The IC₅₀ calculation was performed using GraphPad Prism 7.0, using nonlinear regression.

2.6 Statistical analysis

Results were expressed as the mean of three independent trials $\pm$ standard deviation. All tests were performed in independent triplicate. The interpretation of results was performed by analysis of variance (ANOVA) with Scott-Knott's post-hoc test, where the significance level was set at $p \leq 0.05$. For data analysis, the statistical program R 4.2.1 was used.

3. Results

3.1 *In vitro* inhibition of urease and kinetics mechanisms

Of the 31 Lawsone and lapachol derivatives evaluated, 5 inhibited around 100% of the purified enzyme, 8 inhibited the enzyme activity by more than 50%, as well as the reference inhibitor used (hydroxyurea), 6 derivatives, around 25% and 12 below that level (Figure 2). All inhibitors that showed higher rates of inhibition (PLAU, FLAU, LAW15, LAW16, LAP1) are of the mixed type, that is, they compete with urea for the active site, as well as having the ability to change some allosteric site. Through the Lineweaver-Burk graphs (Figure 3) calculations of the dissociation constants of K_i and K'_i were made.

3.2 Docking molecular

The interaction potential of naphthoquinones at the active site of the urease enzyme of *H. pylori*. Analysis of the amino acid sequences of ureases from different organisms show a high similarity in the structure of these proteins, mainly with regard to the catalytic site (Figure 4). By analyzing the molecular docking poses in the active site region of Urease *H. Pylori*, it can be observed that structures 01 and 02 present a fit in the active site region by their sugar portion and not by their naphthoquinone skeleton (Figure 5). Analyzing in detail the structures 01 and 02 in the active site, it can be observed that the substituent -NAc, present in 02, disturbs the region of the active site, causing steric hindrance with the residue His138. Compound LAW15 can accommodate itself more favorably, which reflects its higher docking score (28.7) (Table 2), compared to LAW16 (23.16) (Figure 6). Molecular docking studies suggest that compound LAP1 would have an unfavorable fit in the active site region, interacting mainly through its naphthoquinone skeleton, but its substituent groups -OAc, from its sugar portion would be uncoupled from this region, (score value equal to 11.24; Figure 7). Compounds PLAU and FLAU, on the other hand, did not present satisfactory complementary fitting points in the region of the active site, since their main point of interaction would be given by their naphthoquinone skeleton, however sterically impeded, which is reflected in their score values, between 17 to 18 sts (Figure 8). Thus, those chemical structures that would have a more favorable fit in the region of the active site of Urease of *H. Pylori* would be those whose catalytic nucleus of interaction

was similar to the -CONHOH group, belonging to the members of the class of urease inhibitors of acetohydroxamic acid derivatives (HAH), whose activity is concentrated in this nucleus -CONHOH (Kobashi, et al., 1966).

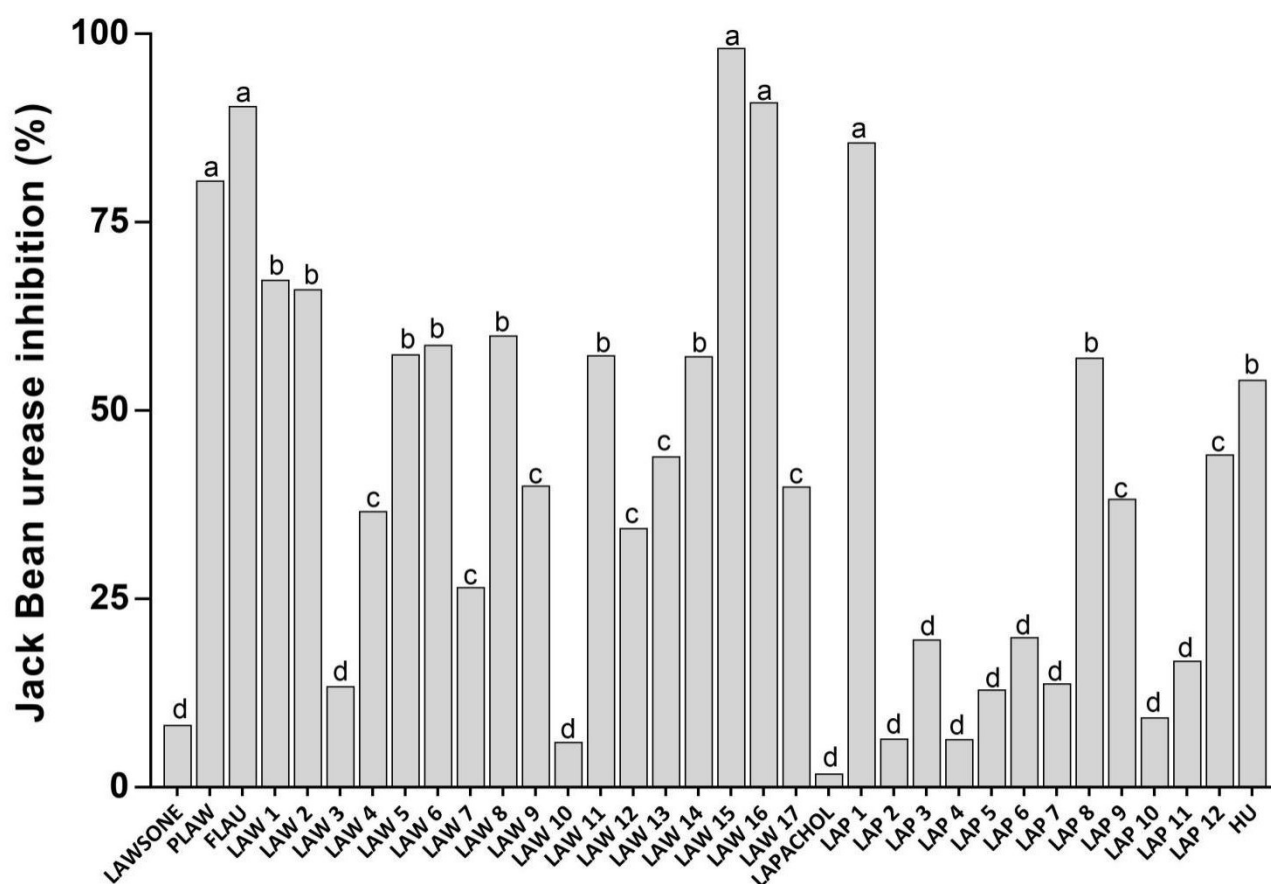


Figure 2. Effect of naftoquinones derivates on the activity of jack bean urease. The enzyme activity was performed from reactions containing 500 μ M compound, 20 mM urea and jack bean type III urease (12.5 mU).

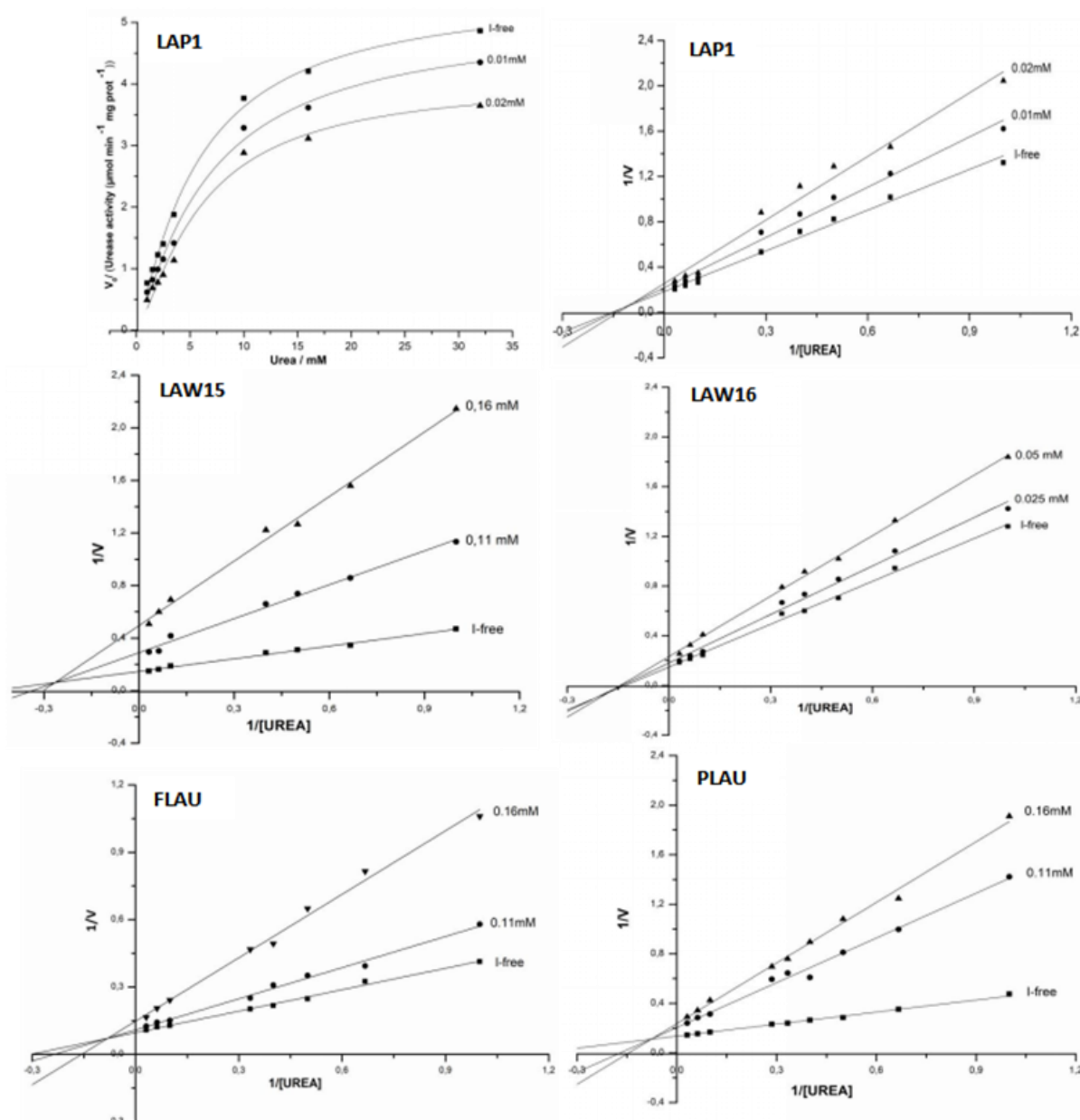


Figure 3. Effect of naphthoquinones derivatives on the activity of jack bean urease. For the screening assay (a), enzyme activity was assessed from reactions containing 1 mM compound-test, 10 mM urea and jack bean type III urease (12.5 mU). As for the kinetics assay, increasing concentrations of urea were incubated for 10 min with urease in the absence of inhibitor (IF) or presence of LAP1, LAW15, LAW16, PLAU and FLAU at indicated concentrations.

Table 1. Effect of naphthoquinones derivatives on the kinetics of jack bean urease. Urea (1 to 32 mM) was incubated for 5 min with jack bean type III urease in the absence or presence of compounds tested at 0.2, 0.4 or 0.8 mM.

Inibidor	Ki	Ki'	IC50	R ²
LAW15	0,05 ± 0,01	0,08 ± 0,03	132,3 ± 4,2	0,99
LAW16	0,18 ± 0,06	0,08 ± 0,03	67,1 ± 2,1	0,99
LAP1	0,03 ± 0,01	0,05 ± 0,01	63,7 ± 4,0	0,98
PLAU	0,17 ± 0,08	0,99 ± 0,7	157,7 ± 7,8	0,99
FLAU	0,04 ± 0,00	0,21 ± 0,01	170,2 ± 10,6	0,99

Tabela 2. Molecular docking data for naphthoquinones derivatives at the active site of *Helicobacter pylori* urease.

Ligand Name	Score	DG
'HAH_m1_5''	40.82	-42.82
'01-LAW15_m1_7'	28.7	-31.73
'02-LAW16_m2_6'	23.16	-40.72
'03-LAP1_m3_1'	11.24	-14.99
'04-PLAU_m4_8'	17.17	-17.79
'05-FLAU_m5_8'	18.2	-18.22

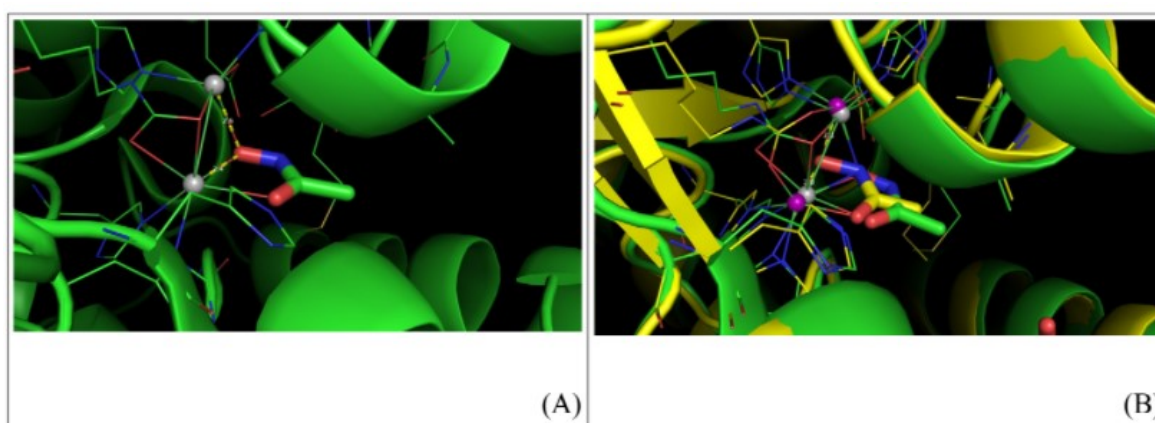


Figure 4. Catalytic site of the urease enzyme: A) *Helicobacter pylori* site showing the carboxylate and histidines, responsible for coordinating the nickel atoms (represented in the form of spheres, in light gray color); B), superimposition of urease enzymes from *Helicobacter pylori* and *Canavalia ensiformis* (jack bean), showing conservation of the catalytic site between these two enzymes.

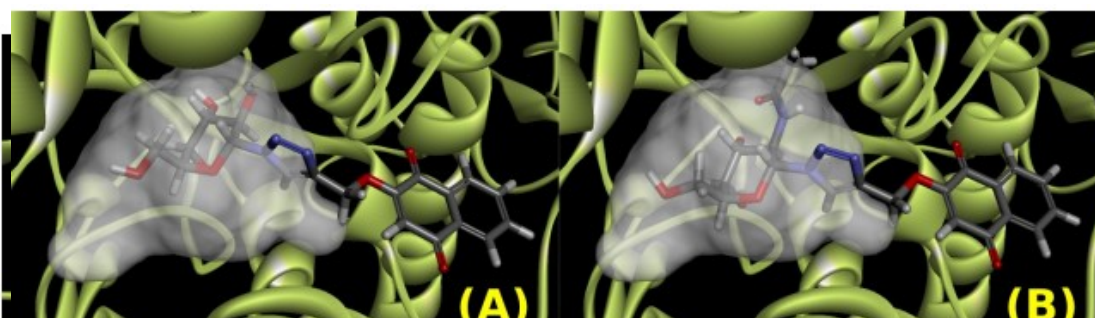


Figure 5. Molecular docking result for 01 (left) and 02 (right), evidencing the fitting of the sugar portion in the active site region.

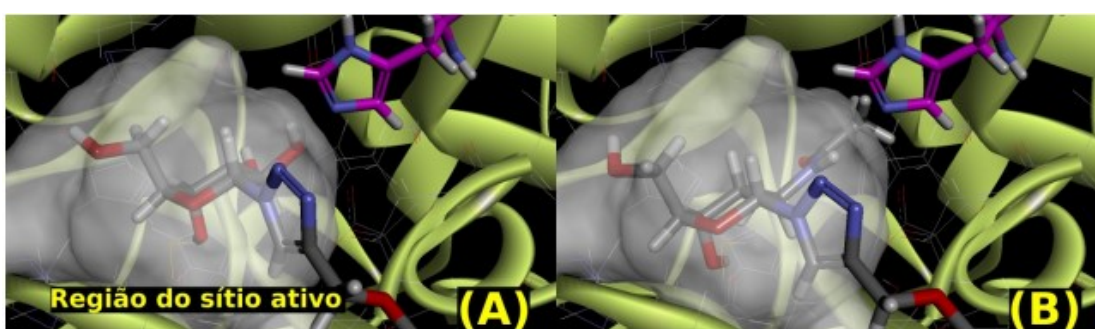


Figure 6. Enlarged active site region, showing the fitting differences in the active site region between 01 and 02, showing the lack of space for the substituent in 02, due to the presence in that region of a Histidine residue.

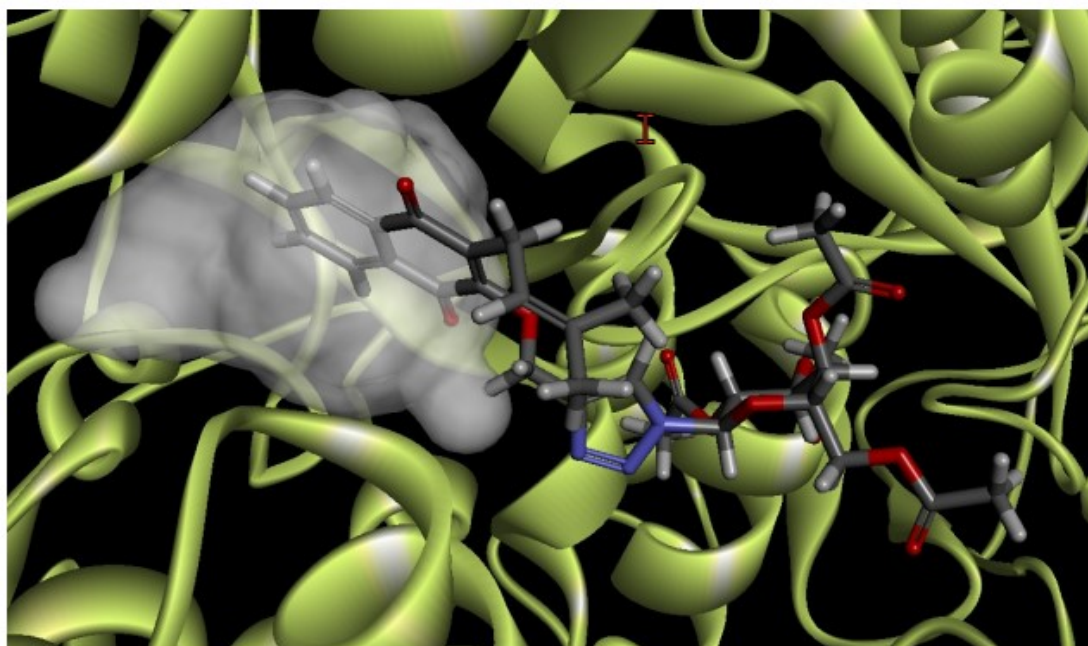


Figure 7. Results of docking of 03, showing the unfavorable fit in the active site region, and the lack of complementary interactions with its chemical structure as a whole.

3.3 Inhibition of *H. pylori* growth

The compounds most effective in inhibiting the growth of *H. Pylori in vitro* were FLAU, LAW16 and LAP1 with IC₅₀ of 64 µg/ml, followed by LAW15 IC₅₀ = 128 µg/ml. The compounds inhibited the growth of *H. pylori*, however, they did not show bactericidal action (Table 3).

Table 3. Minimum inhibitory concentration (MIC) (µg/ml) and minimum bactericidal concentration (MBC) of naphthoquinone derivatives against strain (43504) of *H. pylori*.

Compound	MIC	MBC
PLAU	> 256	-
FLAU	64	-
LAW15	128	-
LAW16	64	-
LAP1	64	-

3.4 Cytotoxicity against AGS

Two of the compounds tested (PLAU and FNQ) exhibited IC₅₀ values lower than those of the reference compound cisplatin (Table 4). Thus, PLAU presented IC₅₀ of 0.43 µg/mL, while FLAU, 2.62 µg/mL. The two triazole glycosides derived from lawsone, which were the compounds that showed strong interaction with the urease active site (Figure 5), had the highest IC₅₀ values, demonstrating little ability to impair AGS cell growth.

Table 4. Effect of naphthoquinones derivatives against the growth of gastric tumor cells.

Compound	IC ₅₀ (µg/ml)
PLAU	0.4269 ± 1.32
FLAU	2.623 ± 1.33
LAW15	36.87 ± 1.13
LAW16	68.83 ± 1.04
LAP1	9.84 ± 0.80
Cisplatin	4.505 ± 0.39

4. Discussion

Quinones are well known for their ability to inhibit ureases (Mazzei et al., 2022). Some studies have also focused on the naphthoquinone class (Rauf et al., 2020). These compounds are well known for their electrophilic capacity and the cytotoxic effect and naphthoquinone derivatives have been widely studied against a number of diseases (Epifano et al, 2014; Ottoni et al, 2020; Mendonça et al., 2022).

All structural modifications in lawsone promoted an increase in inhibition, except for the glycoside LAW3 and the glycoside triazole LAW 10. As the anomers (LAW1, LAW2 and LAW8) showed an average urease inhibition of more than 50%, it can be assumed that the α position of the acetyl group on carbon 4 is a characteristic related to higher percentages of inhibition. Likewise, as with lawsone, better percentages of inhibition with lapachol derivatives occurred with acetylation of the hydroxyl groups on the glycosidic ring, with the α position of the acetyl group on carbon 4.

The glycoside group in the triazole derivative of lawsone interacted well with the active site of the enzyme, a factor responsible for the good score of the compound (LAW15) and (LAW16). Fitting by the quinone portion of the molecules is not favorable with the active site. On the other hand, this same function has a high electrophilic capacity, and its main form of action is the nucleophilic attack of thiol groups, promoting arylation. Quinones can also undergo reductions, originating semi quinones and hydroquinones and originating reactive oxygen species (ROS), which can promote the oxidation of thiols and promote the reversible inactivation of the enzyme. Although lawsone has little ability to produce ROS, inhibition of urease by its 5-hydroxy-1,4-naphthoquinone derivative (juglone) has been linked to the oxidation of the essential urease thiols groups by reactive formation of reactive oxygen species during juglone reduction to semi naphthoquinone (Kot et al., 2010). Jack bean urease has 6 subunits and contains 15 thiols per enzymatic subunit, with a thiol present in the Cys592 residue, located close to the active site and indirectly participating in enzyme catalysis (Krajewska and Zaborska, 2007). Thus, as reactive oxygen species attack both allosteric sites and capable residues directly linked to enzymatic catalysis, the kinetic results corroborate this expected effect.

As naphthoquinones have both the ability to produce ROS and to interact with the active site of the enzyme, the IC_{50} related to the inhibition of the urease enzyme was not proportional to the MIC. For example, compound FLAU showed the highest

IC₅₀ for in vitro inhibition of ureases, but has one of the lowest IC₅₀ for inhibiting *H. pylori* growth. This is due to the dual action of these compounds that have both cytotoxic activity and interact with the enzyme of interest.

The compound (peracetylated β -D-glucoside of lapachol) LAP1 has been studied and its effectiveness against some cancers has been confirmed against HL-60 human leukemia cells (IC₅₀ = 5.7 μ M) (Marques et al, 2020). The same study showed that glycosylation of lapachol was important to reduce the passage of these compounds through the blood-brain barrier and consequently reduce its neurotoxicity. Similarly lawsone derivatives have also been investigated. The same derivatives (LAW2, LAW3 and LAW8) showed activity against breast cancer cell lines (SKBR-3) with IC₅₀ respectively at (2.96, 2.51, 8.86 μ M) (Ottoni et al, 2020). FLAU showed a dual effect on the response against gastric tumoral cells.

5. Conclusions

Although the five selected compounds were excellent urease inhibitors, they had a more potent action as inhibitors of AGS viability. As they scored low on the interaction with the enzyme, the inhibitory effect was shown to be due to the ability to produce ROS and trigger cell toxicity. Despite the high MIC and MBC values, the lawsone derivatives (PLAU and FLAU) are promising compounds that serve for future investigations for the development of new chemicals for the treatment of AGS.

References

- Alba, C., Blanco, A., & Alarcón, T. (2017). Antibiotic resistance in *Helicobacter pylori*. *Current opinion in infectious diseases*, 30(5), 489-497.
- Alipour, M. (2021). Molecular mechanism of *Helicobacter pylori*-induced gastric cancer. *Journal of gastrointestinal cancer*, 52(1), 23-30.
- Badisa, R.B. , Ayuk-Takem, L.T. , Ikediobi, C.O., Walker, E.H. Selective anticancer activity of pure licamichauxiioic-B acid in cultured cell lines, *Pharm. Biol.* 44 (2006) 141–145.
- Crowe, S. E. (2019). *Helicobacter pylori* infection. *New England Journal of Medicine*, 380(12), 1158-1165.
- Epifano F, Genovese S, Fiorito S, Mathieu V, Kiss R. (2014). Lapachol and its congeners as anticancer agents: a review. *Phytochem Rev.* 13(1):37-49. doi: 10.1007/s11101-013-9289-1.
- Debowski, A. W., Walton, S. M., Chua, E. G., Tay, A. C. Y., Liao, T., Lamichhane, B., ...& Benghezal, M. (2017). *Helicobacter pylori* gene silencing in vivo demonstrates urease is essential for chronic infection. *PLoS pathogens*, 13(6), e1006464.
- Fagoonee S, Pellicano R. *Helicobacter pylori*: molecular basis for colonization and survival in gastric environment and resistance to antibiotics. A short review. *Infect Dis Ther.* 2019;51(6):399–408.
- Hu, Y., Zhu, Y., & Lu, N. H. (2017). Novel and effective therapeutic regimens for *Helicobacter pylori* in an era of increasing antibiotic resistance. *Frontiers in Cellular and Infection Microbiology*, 7, Article No. 168.
- Kafarski, P., & Talma, M. (2018). Recent advances in design of new urease inhibitors: A review. *Journal of advanced research*, 13, 101-112.
- Kot, M., Karcz, W., & Zaborska, W. (2010). 5-Hydroxy-1, 4-naphthoquinone (juglone) and 2-hydroxy-1, 4-naphthoquinone (lawsone) influence on jack bean urease activity: elucidation of the difference in inhibition activity. *Bioorganic chemistry*, 38(3), 132-137.
- Krajewska, B., & Zaborska, W. (2007). Double mode of inhibition-inducing interactions of 1, 4-naphthoquinone with urease: arylation versus oxidation of enzyme thiols. *Bioorganic & medicinal chemistry*, 15(12), 4144-4151.
- Marques, L. B., Ottoni, F. M., Pinto, M. C. X., Ribeiro, J. M., de Sousa, F. S., Weinlich, R., ... & Souza-Fagundes, E. M. (2020). Lapachol acetyl glycosylation enhances

- its cytotoxic and pro-apoptotic activities in HL60 cells. *Toxicology in Vitro*, 65, 104772.
- Marshall BJ, Barrett LJ, Prakash C, McCallum RW, Guerrant RL (1990) Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. *Gastroenterology* 99:697–702.
- Mazzei, L., Cianci, M., & Ciurli, S. (2022). Inhibition of urease by hydroquinones: a structural and kinetic study. *Chemistry—A European Journal*.
- Mendonça, D. V., Tavares, G. S., Pereira, I. A., Oliveira-da-Silva, J. A., Ramos, F. F., Lage, D. P., ... & Coelho, E. A. (2022). Flau-A, a naphthoquinone derivative, is a promising therapeutic candidate against visceral leishmaniasis: A preliminary study. *Experimental Parasitology*, 233, 108205.
- Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods* 65 (1983) 55–63.
- Otoni, F. M., Gomes, E. R., Pádua, R. M., Oliveira, M. C., Silva, I. T., & Alves, R. J. (2020). Synthesis and cytotoxicity evaluation of glycosidic derivatives of lawsone against breast cancer cell lines. *Bioorganic & Medicinal Chemistry Letters*, 30(2), 126817.
- Qureshi, N., Li, P., & Gu, Q. (2019). Probiotic therapy in *Helicobacter pylori* infection: a potential strategy against a serious pathogen?. *Applied microbiology and biotechnology*, 103(4), 1573-1588.
- Rauf, A., Aljohani, A. S., Alhumaydhi, F. A., & Naz, S. (2020). A Novel Compound from the Bark of *Diospyros lotus* and their Urease Inhibitory Activity. *Chemistry of Natural Compounds*, 56(6), 1005-1007.
- Ruch TR, Engel JN. Targeting the mucosal barrier: how pathogens modulate the cellular polarity network. *Cold Spring Harb Perspect Biol*. 2017;9(6):a027953.
- Wang, Y. C. (2014). Medicinal plant activity on *Helicobacter pylori*-related diseases. *World Journal of Gastroenterology*, 20(30), 10368–10382.
- Weatherburn, M.W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem*; 39: 971-974.
- Wenceslau, J. P., de Souza, D. F., de Oliveira, M. C., Lemos, T. L., de Sousa, A. L., Trevisan, M. T., & de Mattos, M. C. (2006). Novel lapachol derivatives and their antioxidant activity. *Natural Product Communications*, 1(8), 1934578X0600100812.

Considerações finais

(Tio)hidantoínas derivadas de aminoácidos inibem satisfatoriamente ureases do solo como foi demonstrado no capítulo 1. A partir do primeiro experimento realizado em casa de vegetação, cujos resultados compõem o primeiro capítulo, dois compostos foram selecionados para as próximas análises. Dessa forma, as (tio)hidantoínas, acetilada e não acetilada, derivadas do aminoácido D-triptofano foram selecionadas baseando-se no efeito de aumento da biomassa radicular promovido pela (tio)hidantoína acetilada.

Por meio dos experimentos realizados como biomassa microbiana, respiração basal do solo, não puderam ser detectadas alterações entre o início e o final do experimento em solos tratados ou não com os compostos inibidores. A biomassa microbiana avaliada por meio da técnica de respiração induzida pelo substrato, avalia a comunidade heterotrófica glicolítica e corrobora com a não alteração no metabolismo do carbono previsto por tax4Fun no capítulo 4. As (tio)hidantoínas apresentaram um efeito dependente da concentração. As avaliações de toxicidade focadas em germinação focaram inicialmente, nas altas concentrações dos compostos. No entanto, derivados de D-triptofano demonstraram ter um dual efeito na fisiologia das plantas. Em concentrações que variam de 25 à 100 μM alongaram as raízes de milho, já em concentrações muito baixas próximas à 1 μM estimularam a ramificação da raiz. Dessa forma, as (tio)hidantoínas são moléculas sinalizadoras que exercem seus efeitos apenas quando as plantas são cultivadas na presença do nitrogênio (capítulo 3). Apesar de as (tio)hidantoínas serem promissoras, por terem promovido aumento da performance vegetal, elas exerceram efeito negativo para o ambiente rizosférico ao modificar a composição do microbioma ao longo do tempo e promover a redução da interação modular, o que deixa o microbioma mais vulnerável em relação ao tratamento com NBPT. No entanto outras possibilidades devem ser exploradas, como um possível efeito desses compostos em outros microrganismos do solo, como a comunidade fúngica.

Derivados do lapachol e lausona inibiram satisfatoriamente ureases *in vitro*, como evidenciado no capítulo 5. No entanto, apresentaram inibição no solo de no máximo 20% (Apêndice A). Como o efeito citotóxico de naftoquinonas já é descrito na literatura, esses compostos foram direcionados a avaliações que possibilitarão futuro uso medicinal desses compostos.

Apêndice A

Table 1A. Porcentagem de inibição de ureases do solo por derivados da lausona em latossolo vermelho distrófico.

Composto	LAW1	LAW2	LAW3	LAW4	LAW5	LAW6	LAW7	LAW8	LAW9	LAW10	LAW11	LAW12	LAW13	LAW14	LAW15	LAW16	LAW17
Média de inibição	-0,24	2,71	2,63	-9,77	-3,22	-4,59	17,57	-6,75	-13,72	27,40	13,35	7,86	-18,77	-13,80	14,36	-20,93	9,4
Desvio padrão	13,6	30,2	15,1	17,3	11,6	9,4	12,5	15,8	25,4	17,2	15,3	11,3	13,6	9,6	8,1	14,5	12,8

Valores representam média e desvio padrão de três experimentos independentes com compostos à 500 µM.

Table 2A. Porcentagem de inibição de ureases do solo por derivados do lapachol em latossolo vermelho distrófico.

Composto	LAP1	LAP2	LAP3	LAP4	LAP5	LAP6	LAP7	LAP8	NBPT
Média de inibição	-0,50	15,04	-7,24	19,82	-11,22	-25,61	-21,14	-19,09	39,46
Desvio padrão	38,5	7,4	20,5	11,9	23,0	16,5	20,9	21,1	6,9

Valores representam média e desvio padrão de três experimentos independentes com compostos à 500 µM.