UNIVERSIDADE ESTADUAL DE PONTA GROSSA

SETOR DE CIÊNCIAS AGRÁRIAS E DE TECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

FERNANDA FURMAM CHEROBIM

CO-INOCULATION OF *Azospirillum brasilense* STRAINS AND *Bradyrhizobium* sp. IN SOYBEAN CROPS AND DEVELOPMENT OF A NEW TECHNOLOGY OF APPLICATION

CO-INOCULAÇÃO DE ESTIRPES DE *A. brasilense* E *Bradyrhizobium* sp. NA CULTURA DA SOJA E O DESENVOLVIMENTO DE UMA NOVA TECNOLOGIA DE APLICAÇÃO

PONTA GROSSA

2023

FERNANDA FURMAM CHEROBIM

CO-INOCULATION OF *Azospirillum brasilense* STRAINS AND *Bradyrhizobium* sp. IN SOYBEAN CROPS AND DEVELOPMENT OF A NEW TECHNOLOGY OF APPLICATION

CO-INOCULAÇÃO DE ESTIRPES DE *A. brasilense* E *Bradyrhizobium* sp. NA CULTURA DA SOJA E O DESENVOLVIMENTO DE UMA NOVA TECNOLOGIA DE APLICAÇÃO

Thesis presented to Universidade Estadual de Ponta Grossa for obtain the Doctor (PhD) title in Agronomy – Concentration: Soil Science and Environmental Resources. Research area: Use and Management of Natural Resources.

Advisor: Prof. Dra. Carolina Weigert Galvão

PONTA GROSSA

2023

Cherobim, Fernanda Furmam
C522 Co-inoculation of *Azospirillum brasilense* strains and *Bradyrhizobium* sp. in soybean crops and development of a new technology of application / Fernanda Furmam Cherobim. Ponta Grossa, 2023.
65 f.

Tese (Doutorado em Agronomia - Área de Concentração: Agricultura), Universidade Estadual de Ponta Grossa.

Orientadora: Profa. Dra. Carolina Weigert Galvão.

1. Biological nitrogen fixation. 2. Nodulation. 3. New technologies. 4. Hydrogel. 5. Biotechnology. I. Galvão, Carolina Weigert. II. Universidade Estadual de Ponta Grossa. Agricultura. III.T.

CDD: 633

Ficha catalográfica elaborada por Maria Luzia Fernandes Bertholino dos Santos- CRB9/986

CERTIFICADO DE APROVAÇÃO

Título da Tese: **"Co-inoculation of *Azospirillum brasilense* strains and *Bradyrhizobium* sp. in soybean crops and development of a new technology of application"**

Nome: **Fernanda Furmam Cherobim**

Orientador: **Profª. Drª. Carolina Weigert Galvão**

Aprovado pela Comissão Examinadora:

Profª. Drª. Carolina Weigert Galvão

Prof. Dr. Eduardo Fávero Caires

Prof. Dr. Daniel Ruiz Potma Gonçalves

Prof. Dr. Vandeir Francisco Guimarães

Prof. Dr. Emanuel Maltempi de Souza

Data da Realização: 19 de abril de 2023.

sei! assinatura eletrônica — Documento assinado eletronicamente por **Carolina Weigert Galvao, Professor(a)**, em 02/05/2023, às 11:37, conforme Resolução UEPG CA 114/2018 e art. 1º, III, "b", da Lei 11.419/2006.

sei! assinatura eletrônica — Documento assinado eletronicamente por **Daniel Ruiz Potma Goncalves, Professor(a)**, em 02/05/2023, às 15:58, conforme Resolução UEPG CA 114/2018 e art. 1º, III, "b", da Lei 11.419/2006.

Documento assinado eletronicamente por **Eduardo Favero Caires**, **Professor(a)**, em 04/05/2023, às 11:01, conforme Resolução UEPG CA 114/2018 e art. 1º, III, "b", da Lei 11.419/2006.

A autenticidade do documento pode ser conferida no site https://sei.uepg.br/autenticidade informando o código verificador **1416709** e o código CRC **7916C75D**.

23.000020565-0 1416709v5

Documento assinado digitalmente
VANDEIR FRANCISCO GUIMARAES
Data: 30/06/2023 16:04:21-0300
Verifique em https://validar.iti.gov.br

Documento assinado digitalmente
EMANUEL MALTEMPI DE SOUZA
Data: 29/06/2023 14:11:51-0300
Verifique em https://validar.iti.gov.br

To my Family for being my first and eternal teachers,

I dedicate.

ACKNOWLEDGMENTS

To God, for the gift of life and for granting me the necessary gifts to carry out this work.

To all my family, for always praying for me, encourage me and being my emotional support. Especially to my husband Jordano, my grandparents, my parents Orizes and Leni and my brother Pedro, you are my strength. Thanks for so much.

To “Universidade Estadual de Ponta Grossa” (UEPG), for all the support, especially to the Graduate Program in Agronomy.

To “Coordenação de aperfeiçoamento pessoal de nível superior” (CAPES) for the fellowship that allowed project development.

To my advisors, Profa. Dra. Carolina W. Galvão e Prof. Dr. Rafael M. Etto for the guidance, advice, teachings and friendship developed during a long time together.

To all the friends of the "Laboratório de Biologia Molecular Microbiana" (LABMOM), for all their help, companionship and assistance in carrying out the analyses. I won't name names because there are so many. But know that you were very important for the realization of this work and you will always be in my heart. Gratitude.

To all my graduate colleagues, for the opportunity for professional and personal growth.

The LABMOM technician, Lígia Dombroski dos Santos, for all her friendship, attention, help, encouragement and prayers.

For all the people who were not mentioned here, but who were fundamental in my formation, directly or indirectly, contributing to my professional and personal growth.

I can do all things through Christ who strengthens me (Philippians 4:13).

ABSTRACT

CHEROBIM, F. F. **Co-inoculation of A*zospirillum brasilense* strains and *Bradyrhizobium* sp. in soybean crops and development of a new technology of application.** Advisor: Carolina Weigert Galvão. Ponta Grossa, 2023.Thesis (Doctorate in Agronomy) – Universidade Estadual de Ponta Grossa, Ponta Grossa, 2023.

The success of soybeans, in large part, is justified by the benefits arising from the inoculation of seeds with *Bradyrhizobium japonicum*, an association capable of supplying the demand for nitrogen by the crop, since these bacteria supply nitrogen to the plants through biological nitrogen fixation (BNF). A new technology has been increasingly adopted by soybean producers, called co-inoculation, which aims to promote plant growth by combining different mechanisms of different microorganisms. In the case of soybeans, co-inoculation occurs with the mixed inoculation of *B. japonicum* and/or *B. elkanii* and *A. brasilense* AbV5/AbV6. Seeking to enhance even more the positive responses with the co-inoculation in soybean, this work aims to test new strains of *A. brasilense* in the co-inoculation of the soybean crop, under field conditions. Three new strains, *A. brasilense* HM053, HM210 and IH1 were evaluated. *A. brasilense* HM053 and HM210 which excrete ammonium constitutively, and *A. brasilense* IH1 which is more resistant to oxidative stress. The experiments were carried out in the cities of Dois Vizinhos, Mandaguaçu, Rio Verde, Indiara and Pindorama, seeking different soil and climate conditions in Brazil to evaluate soybean growth and productivity improvement under different conditions. Co-inoculation with the new strains showed promising results for the characteristics evaluated, sometimes being statistically equal to the use of the AbV5/6 strain, sometimes being superior. At Mandaguaçu and Dois Vizinhos, in the flowering period, the HM053 strain stood out, presenting values higher than those found with the AbV5/6 strain, for the parameters of nodulation, dry weight and nitrogen content of the aerial part. However, it is notable that there is a lack of co-inoculation response pattern in the different sites evaluated. Therefore, a study in order to improve the efficiency of the inoculation was developed, with the objective of encapsulating the bacteria, protecting them from biotic and abiotic stresses, thus increasing the viability and stability of the bacterial cells. The *A. brasilense* AbV5/6 strain was encapsulated in a double cross-linked hydrogel granule based on cationic starch, using the swelling diffusion method followed by desiccation. The survival evaluation of *A. brasilense* AbV5/6 was carried out up to sixty days after loading and storage, and the average of viable bacteria remained in the order of magnitude of 10^7 CFU g, indicating that the hydrogel spheres are adequate to protect the *A. brasilense* for a period of at least 60 days. In addition, an experiment was carried out in a plant growth chamber and greenhouse to observe the effects of inoculation of encapsulated bacteria in corn seeds. In a growth chamber, the association of the inoculation with the hydrogel showed increments of 17% in the fresh weight of the aerial part and of 19% in the length of the roots. In the greenhouse, inoculation with *A. brasilense* AbV5/6 increased the levels of chlorophyll a and b, however, only in the presence of the hydrogel. The presence of the hydrogel improved the fresh weight of shoots by 6% and the height of corn plants by 7%. The results showed that this technology can enhance the effects of *A. brasilense* AbV5/6 in promoting maize growth under controlled conditions. More studies must be carried out to validate this technology in other crops and under field conditions.

Key-words: Plant growth-promoting bacteria, biological nitrogen fixation, hydrogel, sustainable agriculture, bacteria encapsulation.

RESUMO

CHEROBIM, F. F. **Co-inoculação de estirpes de *Azospirillum brasilense* e *Bradyrhizobium* sp. na cultura da soja e desenvolvimento de uma nova tecnologia de aplicação.** Orientador: Carolina Weigert Galvão. Ponta Grossa, 2023.Tese (Doutorado em Agronomia) – Universidade Estadual de Ponta Grossa, Ponta Grossa, 2023.

O sucesso da soja, em grande parte, é justificado pelos benefícios oriundos da inoculação das sementes com *Bradyrhizobium japonicum*, associação capaz de suprir a demanda de nitrogênio pela cultura, visto que estas bactérias fornecem nitrogênio para as plantas através da fixação biológica de nitrogênio (FBN). Uma nova tecnologia vem sendo cada vez mais adotada pelos produtores de soja, denominada de co-inoculação, a qual tem por objetivo promover o crescimento das plantas combinando mecanismos distintos de diferentes microrganismos. No caso da soja, a co-inoculação se dá, na maioria das vezes, com a inoculação mista de *B. japonicum* e/ou *B. elkanii* e *A. brasilense* AbV5/AbV6. Buscando potencializar ainda mais as respostas positivas com a co-inoculação em soja, este trabalho tem por objetivo testar novas estirpes de *A. brasilense* na co-inoculação da cultura da soja, em condições de campo. Três novas linhagens, *A. brasilense* HM053, HM210 e IH1 foram avaliadas. *A. brasilense* HM053 e HM210 que excretam amônia constitutivamente e o *A. brasilense* IH1 que é mais resistente ao estresse oxidativo. Os experimentos foram realizados nas cidades de Dois Vizinhos, Mandaguaçu, Rio Verde, Indiara e Pindorama., buscando condições distintas de solo e clima no Brasil para avaliar o crescimento da soja e a melhoria da produtividade em diferentes condições. A co-inoculação com as novas estirpes apresentaram resultados promissores para as características avaliadas, ora sendo iguais estatisticamente ao uso da estirpe AbV5/6, ora sendo superiores. Em Mandaguaçu e Dois Vizinhos, no período de florescimento, a estirpe HM053 foi destaque, apresentando valores superiores ao encontrado com a estirpe AbV5/6, para os parâmetros de nodulação, peso seco e teor de nitrogênio da parte aérea. Contudo, é notável que existe uma falta de padrão de resposta da co-inoculação nos diferentes locais avaliados. Por isso, um estudo a fim de melhorar a eficiência da inoculação foi desenvolvido, com o objetivo de encapsular as bactérias, protegendo-as de estresses bióticos e abióticos, aumentando assim, a viabilidade e estabilidade das células bacterianas. A estirpe *A. brasilense* AbV5/6 foi encapsulada em um grânulo de hidrogel reticulado duplo baseado em amido catiônico, através do método de difusão por intumescimento seguido de dessecação. A avaliação de sobrevivência de *A. brasilense* AbV5/6 foi realizada até sessenta dias após o carregamento e armazenagem, e a média de bactérias viáveis, permaneceu na ordem de grandeza de 10^7 UFC g, indicando que as esferas de hidrogel são adequadas para proteger o *A. brasilense* por um período de pelo menos 60 dias. Além disso, foi realizado um experimento em câmara de crescimento vegetal e casa de vegetação para observar os efeitos da inoculação de bactérias encapsuladas em sementes de milho. Em câmara de crescimento, a associação da inoculação com o hidrogel, apresentou incrementos de 17% no peso fresco da parte aérea e de 19% no comprimento das raízes. Em casa de vegetação, a inoculação com *A. brasilense* AbV5/6 aumentou os teores de clorofila a e b, porém, somente na presença do hidrogel. A presença do hidrogel melhorou em 6% o peso fresco de parte aérea e em 7% a altura das plantas de milho. Os resultados mostraram que esta tecnologia pode potencializar os efeitos de *A. brasilense* AbV5/6 na promoção do crescimento de milho em condições controladas. Mais estudos devem ser realizados para validar essa tecnologia em outras culturas e em condições de campo.

Palavras-chave: bactérias promotoras do crescimento vegetal, fixação biológica de nitrogênio, hidrogel, agricultura sustentável, encapsulamento de bactérias.

FIGURES LIST

TABLES LIST

SUMMARY

PRESENTATION

This thesis was written according to “Manual de normalização bibliográfica para trabalhos científicos, UEPG (2019)” and is composed of the following chapters:

- Evaluation of *A. brasilense* new strains in soybean co-inoculation, in submission process.

- *Azospirillum brasilense* AbV5/6 encapsulation in dual-crosslinked beads based on cationic starch, published in Carbohydrate Polymers journal, volume 308 (2023), doi.org/10.1016/j.carbpol.2023.120631.

All the chapters reproduce literally the text submitted to the journals and despite interconnections, one chapter is independent of the others (the read of any chapter don’t presume the read of the others and the acronyms, equations, figures, tables and references refers only to the current chapter). Preceding the first chapter there is a general introduction and the last item is composed by the general conclusions.

GENERAL INTRODUCTION

The total production of grains in Brazil is approximately 310 million tons, with soybean and corn being the main producers, with a production of approximately 152 and 125 million tons respectively, in the 2022/23 harvest (CONAB, 2023). Increasing agricultural production sustainably to maintain the food security of a growing global population is one of the significant challenges facing modern agriculture, making it clear the need to seek new approaches to maximize crop productivity (MUKHERJEE, 2022).

The use of Plant Growth Promoting Bacteria (PGPB) is a promising alternative to aid plant development and increase productivity, as they regularly establish mutualistic interactions with host plants related to nutrient uptake (biological nitrogen fixation - BNF, solubilization of phosphorus and potassium), greater resistance to biotic and abiotic stress, regulation of plant development through the production of compounds, including phytohormones and specific signaling compounds between organisms (SHAH et al., 2021).

The use of microbial inoculants or biofertilizers are products containing live microorganisms, which have the ability to establish different types of association with plants, being a sustainable alternative to mineral fertilizers (SANTOS; NOGUEIRA; HUNGRIA, 2021). Rhizobia are symbiotic diazotrophic bacteria, capable of forming a close relationship with the host plant, forming specific structures, the nodules, where the process of biological nitrogen fixation (BNF) occurs (ORMEÑO-ORRILLO; HUNGRIA; MARTINEZ-ROMERO, 2013). This symbiosis occurs mainly with plants belonging to the Fabaceae family, in the case of soybean with *Bradyrhizobium* spp. and in the case of common bean, with *Rhizobium* spp. (HUNGRIA; MENNA; DELAMUTA, 2015).

The most successful case with the contribution of BNF in Brazil is represented by the soybean crop, where the use of inoculants from the 1960s onwards ensured the competitiveness of the crop when compared to production in other countries, directly reflecting on the trade balance of the country (EMBRAPA, 2022). Due to the importance of the soybean crop for Brazil and the benefits of co-inoculating with *Azospirillum*, this technology becomes interesting and promising to improve crop performance, since its use improves soybean yield, without the addition of nitrogen-based mineral fertilizers, thus helping to reduce costs and follow current sustainability practices in agriculture (HUNGRIA; NOGUEIRA; ARAUJO, 2015).

For maize, inoculation with *A. brasilense* AbV5 and AbV6 was recommended from 2010 in Brazil (HUNGRIA et al., 2010) and since then many studies have been carried out on this topic. The increase in corn yield promoted by seed inoculation is attributed to the production of phytohormones by bacteria (FUKAMI et al., 2017). HM053 is also showing to be a promising *A. brasilense* strain. It is a spontaneous mutant of glutamine synthetase (GS), which has the ability to excrete ammonium and fix nitrogen constitutively, even in the presence of high concentration of NH_4^+ (MACHADO et al., 1991). *A. brasilense* HM053 is derived from the *A. brasilense* Sp7 strain (MACHADO et al., 1991), which also gave rise to the currently recommended *A. brasilense* strains AbV5 and AbV6.

Pankievicz et al. (2015) reported that *Setaria viridis* inoculated with the mutant strain *A. brasilense* HM053 was able to fix approximately 12,231 parts per trillion of nitrogen on a root dry mass basis, demonstrating that under suitable conditions *Setaria viridis* can obtain enough nitrogen to supply its daily demand through BNF and promote plant growth. In wheat, the inoculation of the HM053 strain was able to colonize the surface of the roots and increase the dry weight of the aerial part and the root by 30 and 49%, respectively, when compared to non-inoculated plants and by 30 and 31% when compared to the parental strain FP2 (SANTOS et al., 2017). Furthermore, strain HM053 expressed the *nifH* gene at a level 278 times higher than the parental strain in wheat (SANTOS et al., 2017).

Pedrosa et al. (2019) evaluated the effect of inoculation with *A. brasilense* HM053 and *A. brasilense* AbV5 on the corn crop in four field experiments and found that grain yield was higher with strain HM053 in three experiments compared to inoculation with the strain AbV5 and that strain HM053 provided an increase in production 12 between 4.7 to 29% or 460.5 to 1769.3 kg ha^{-1}, showing that this strain is a new alternative for a more sustainable agriculture.

The results of a meta-analysis demonstrated the lack of a positive and significant contribution of co-inoculation to soybean grain yield, however, indirect evidence reports that the identification of bacterial strains that cause complementary effects on plant development is a crucial step for the development of more efficient inoculants for the soybean crop (ZEFFA et al., 2020).

In addition, there are some problems regarding the colonization and persistence of the inoculant after its introduction into the competitive soil environment, such as attack by bacteriophages or protozoa (SIVAKUMAR; PARTHASARTHI; LAKSHMIPRIYA, 2014).

Therefore, it is essential to enable the survival of microorganisms introduced into the soil and increase their availability for crops (SZOPA et al., 2022).

According to Bashan et al. (2014), there are four factors that influence the success of the inoculation: the growth phase at the time of mixing the bacterial culture with a carrier, the rate of drying and rehydration, appropriate characteristics of the carrier material and inoculation technology.

In order to improve the efficiency of seed inoculation, other inoculation technologies have been studied, such as encapsulation, whose objective is to stabilize cells, protect against exposure to biotic and abiotic stresses and potentially increase the viability and stability of bacterial cells during the production and storage of strains important for agriculture, being one of the most recent and efficient techniques for the protection of beneficial bacteria against unfavorable soil conditions (RISEH et al., 2021).

Encapsulation can be performed through the use of hydrogel, which is designed to create a temporary barrier to microorganisms and provide a microenvironment that supports their viability, where their matrix is degraded due to bacterial activity in the soil, ensuring a slow release of the inoculant in the soil, thus increasing its efficiency for a longer period (SZOPA et al., 2022). Hydrogel production is mainly made by natural biopolymers, such as alginate, cellulose, starch, chitin, lignin or gelatin (MALUSÁ; SAS-PASZT; CIESIELSKA, 2012).

Brazil has a long tradition in research with inoculants based on rhizobia and *Azospirillum* and also on legislation for quality control of inoculants (BOCATTI et al., 2022). Therefore, this work aimed to evaluate the efficiency of new strains of *A. brasilense* in the co-inoculation of the soybean crop and to validate a new inoculant application technology, via physical hydrogel, in in vitro and greenhouse tests, using the corn crop.

REFERENCES

BASHAN, Y. et al. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). **Plant and Soil**, v. 378, p.1-33, 2014.

BOCATTI, C. R. et al. Microbiological quality analysis of inoculants based on *Bradyrhizobium* spp. and *Azospirillum brasilense* produced “on farm” reveals high contamination with non-target microorganisms. **Brazilian Journal of Microbiology**, v. 53, p. 267-280, 2022.

CONAB – Companhia Nacional de Abastecimento. **Acompanhamento da Safra Brasileira de Grãos**. Brasília, v. 10, p. 47-57, Safra 2022/23, n. 4 – Quarto Levantamento, janeiro, 2023.

EMBRAPA. **Fixação biológica de nitrogênio**. Disponível em:<https://www.embrapa.br/tema-fixacao-biologica-de-nitrogenio/nota-tecnica>. Acesso em: 06 dezembro 2022.

FUKAMI, J. et al. Phytohormones and induction of plant-stress tolerance and defense genes by seed and foliar inoculation with *Azospirillum brasilense* cells and metabolites promote maize growth. **AMB Express**, v. 7, nº, 153, 2017.

HUNGRIA M. et al. Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. **Plant Soil**, v. 331, p. 413–425, 2010

HUNGRIA, M.; MENNA, P.; DELAMUTA, J.R.M. *Bradyrhizobium*, the ancestor of all rhizobia: phylogeny of housekeeping and nitrogen-fixation genes. In: DE BRUIJN, F.J.; editor. **Biological nitrogen fixation**. New Jersey: John Wiley & Sons Inc, 2015. p. 191-202.

HUNGRIA, M.; NOGUEIRA, M. A.; ARAUJO, R. S. Soybean Seed Co-Inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*: A New Biotechnological Tool to Improve Yield and Sustainability. **American Journal of Plant Sciences**, v. 6, p. 811-817, 2015.

MACHADO, H. B. et al. Excretion of ammonium by Azospirillum brasilense mutants resistant to ethylenediamine. **Can J Microbiol.**, v. 37p. 549–553, 1991.

MALUSÁ, E.; SAS-PASZT, L.; CIESIELSKA, J. Technologies for Beneficial Microorganisms Inocula Used as Biofertilizers. **The Scientific World Journal**, v. 2012, p. 491206-12, 2012.

MUKHERJEE, ARIJIT. What do we know from the transcriptomic studies investigating the interactions between plants and plant growth-promoting bacteria? **Frontiers in Plant Science**, v. 13, 2022.

ORMEÑO-ORRILLO, E.; HUNGRIA, M.; MARTINEZ-ROMERO, E. Dinitrogen-fixing prokaryotes. In ROSENBERG, E.; DELONG, E. F.; LORY, S.; STACKEBRANDT, E.; THOMPSON, F. editors. **The prokaryotes: prokaryotic physiology and biochemistry**. Berlin Heidelberg: Springer, 2013. p. 427-51.

PANKIEVICZ, V. C. S. et al. Robust biological nitrogen fixation in a model grass-bacterial association. **Plant Journal**, v. 81, p. 907–919, 2015.

PEDROSA, F. O. et al. The ammonium excreting *Azospirillum brasilense* strain HM053: a new alternative inoculant for maize. **Plant and Soil**, v. x, p. 1-12, 2019.

RISEH, R. S. Encapsulation of Plant Biocontrol Bacteria with Alginate as a Main Polymer Material. **International Journal of Molecular Sciences**, v. 22, p. 11165, 2021.

SANTOS, K.F.D.N. et al. Wheat colonization by an *Azospirillum brasilense* ammoniumexcreting strain reveals upregulation of nitrogenase and superior plant growth promotion. **Plant and Soil**, v. 415, p. 245-255, 2017.

SANTOS, M. S.; NOGUEIRA, M. A.; HUNGRIA, M. Outstanding impact of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 on the Brazilian agriculture: Lessons that farmers are receptive to adopt new microbial inoculants. **Revista Brasileira de Ciência do Solo**, v. 45, e0200128, 2021.

SIVAKUMAR, P. K.; PARTHASARTHI, R.; LAKSHMIPRIYA, V. P. Encapsulation of plant growth promoting inoculant in bacterial alginate beads enriched with humic acid. **Int.J.Curr.Microbiol.App.Sci**., v. 3, p. 415-422, 2014.

SHAH, A. et al. PGPR in Agriculture: A Sustainable Approach to Increasing Climate Change Resilience. **Front. Sustain. Food Syst.**, v. 5, 2021.

SZOPA, D. et al. Encapsulation efficiency and survival of plant growth-promoting microorganisms in an alginate-based matrix – A systematic review and protocol for a practical approach. **Industrial Crops & Products**, v. 181, p.114846, 2022.

ZEFFA, D. M. et al. Effects of plant growth-promoting rhizobacteria on co-inoculation with Bradyrhizobium in soybean crop: a meta-analysis of studies from 1987 to 2018. **PeerJ.**, v. 8, p. e7905, 2020.

CHAPTER 1 – EVALUATION OF *A. brasilense* NEW STRAINS IN SOYBEAN CO-INOCULATION

Abstract: The inoculation of *Bradyrhizobium* spp. in soybean is a widespread technology in Brazil and one of the most successful cases of plant-bacteria interaction once all the nitrogen required by the plant is provided through biological nitrogen fixation. However, further studies are needed to test new strains and fit them to new soybean varieties. The co-inoculation of *Bradyrhizobium* spp. with *Azospirillum brasilense* AbV5/6 started to be recommended in 2013 in Brazil, motivated by the combination of different diverse mechanisms to improve plant development provided by the two bacteria. However, the adoption of this technology by farmers is still modest once the yield gain varies. Since *A. brasilense* strain IH1 has great resistance to oxidative and the strains HM053 and HM210 fix nitrogen even in the presence of ammonium, we co-inoculated *Bradyrhizobium* spp. with these new strains of *A. brasilense* in soybean. The experiments were carried out in distinctive soil and climate conditions in Brazil to evaluate the soybean growth and yield improvement. The co-inoculation with HM053 strain increased the number and weight of nodules and improved the shoot growth and grain productivity. This promising technology generates environmental and economic gains, since it substitutes nitrogenous fertilizers, increase yield, and contributes for a sustainable agriculture.

Key Words: Biological nitrogen fixation, nodulation, productivity, new technologies.

1.1 INTRODUCTION

The soybean crop is a vital component of Brazilian agriculture, with over 70% of its total production exported, making Brazil the leading exporter of soybeans and the second-largest exporter of soy meal and soybean oil in the world (ALI et al., 2022). The success of soybean cultivation in Brazil can be attributed to the inoculation of seeds with *Bradyrhizobium* bacteria, which can fully satisfy the crop's nitrogen demand, resulting in high grain yields (HUNGRIA; NOGUEIRA; ARAUJO, 2015).

The symbiotic relationship between *Bradyrhizobium* and soybean is considered one of the most important natural relationships utilized in agricultural activities (ZEFFA et al., 2020). *Bradyrhizobium*'s nitrogen fixation can increase grain productivity, eliminating or reducing reliance on inorganic nitrogen fertilizers in cultivation, and saving up to US$20 billion annually on nitrogen fertilizers (SANTOS; NOGUEIRA; HUNGRIA, 2019).

In recent years, mixed inoculants have become more popular as they promote plant growth through different mechanisms of various microorganisms (SANTOS; NOGUEIRA; HUNGRIA, 2019). Since 2013, co-inoculation of *A. brasilense* and *Bradyrhizobium* spp. has been recommended in Brazil's soybean crop (SANTOS; NOGUEIRA; ARAUJO, 2013). Currently, around 106 inoculants containing *Azospirillum* as the active ingredient are produced in South America, with *A. argentinense* Az39 (formerly *A. brasilense* Az39) being emphasized in Argentina and strains of *A. brasilense* AbV5/6 in Brazil (CASÁN et al. 2020). Besides AbV5/AbV6, other *A. brasilense* Sp7 derivatives strains have shown great potential (MACHADO et al. 1991; HIGUTI; PEDROSA, 1985). Strains HM210 and HM053 are spontaneous mutants of glutamine synthetase (GS), which have the ability to excrete ammonium and fix nitrogen constitutively, even in the presence of high concentrations of NH_4^+ (MACHADO et al. 1991). IH1 strain is resistant to high concentrations of H_2O_2 due to the presence of high levels of catalase, thus promoting greater resistance to oxidative stress (HIGUTI; PEDROSA, 1985).

A. brasilense HM053 showed to supply *Setaria viridis* daily demand of nitrogen through BNF under controlled conditions (PANKIEVICZ et al. 2015). In wheat and barley, the inoculation of the HM053 strain was able to colonize the surface of the roots and increase biometric parameters of the plants (SANTOS et al. 2017a and 2017b). Besides, HM053 strain provided an increase in maize grain yield between 4.7 to 29% or 460.5 to 1769.3 kg/ha, showing that this strain is a new alternative for a more sustainable agriculture (PEDROSA et al. 2019).

A. brasilense HM210 strain was tested in the beet culture, showing superior results for the diameter and length of tuberous roots (SOUSA, 2022).

The ability of *Azospirillum* spp. to synthesize phytohormones and stimulate plant growth, especially the root system, can favor nodulation and biological nitrogen fixation performed by *Bradyrhizobium* when they are associated (PRANDO et al., 2019). Co-inoculation with *A. brasilense* promotes more abundant nodulation as a result of an increase in the number of sites of infection, which are the root hairs, from where the bacteria enters and starts nodule formation (TIMMERS, 2000). Co-inoculation of *Bradyrhizobium* spp. with *A. brasilense* AbV5/AbV6 increased by 7.7 to 20.3% of the soybean grain yield when compared to the single inoculation (HUNGRIA, NOGUEIRA and ARAUJO, 2013; GALINDO et al., 2018; FERRI et al., 2017). Despite the benefits of co-inoculation for rural producers, only 29% of soybean crops use this technology nationally, however, the percentage of use of *A. brasilense* has been increasing each year in important agricultural crops, such as soybeans and maize (ANPII, 2023).

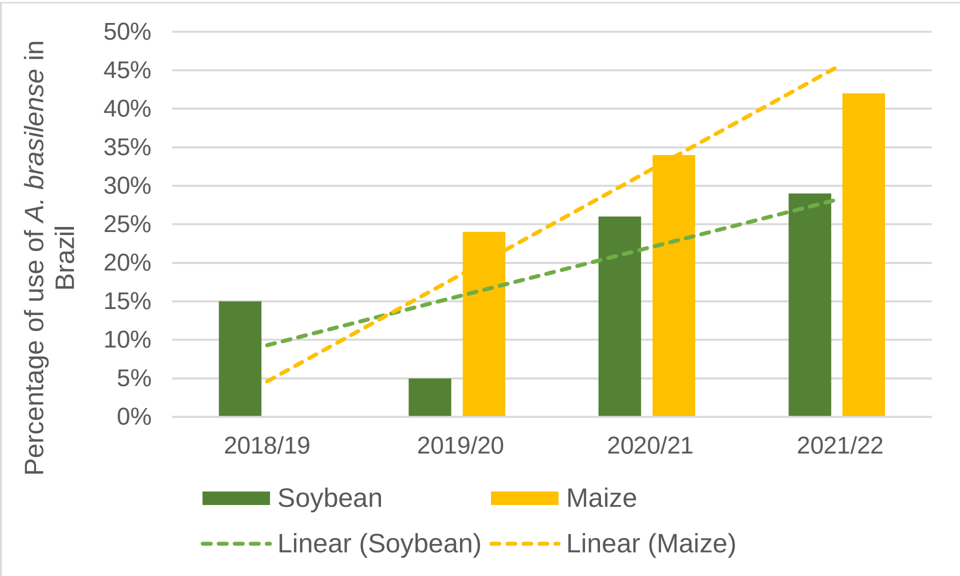

Figure 1.1 - Percentage of use of *A. brasilense* in soybean and maize crops in Brazil.

In this study, we report the results of experiments carried out in different edaphoclimatic conditions in Brazil, exploring the co-inoculation of soybean with three new strains of *A. brasilense* (IH1, HM053 and HM210). We evaluated parameters of nodulation and productivity of soybean plants.

1.2 MATERIAL AND METHODS

1.2.1 Inoculants and experimental design

This experiment was carried out in 2019/20, in the soybean crop, with the participation of ANPII - National Association of Inoculant Producers and Importers. The new strains of *A. brasilense*, IH1, HM053, and HM210, are deposited at the Federal University of Paraná – UFPR, in the Department of Biochemistry and Molecular Biology (Curitiba, Paraná). ANPII partner companies were responsible for standardizing and production of each new strain inoculants, as well as for supplying the commercial inoculants regulated in Brazil, *A. brasilense* AbV5/6 and *Bradyrhizobium* SEMIA 5079 (*B. japonicum*) and SEMIA 5080 (*B. diazoefficiens*). All *A. brasilense* strains were standardized to a concentration of 2 to 7.8 x 10^8 CFU/mL and the *Bradyrhizobium* inoculant to a concentration of 5 x 10^9 CFU/mL. The recommended dose for both *Bradyrhizobium* and *A. brasilense* was 100 ml ha^{-1}.

Seed treatment was carried out with pyraclostrobin + methyl thiophanate + fipronil, at a dose of 2 mL kg^{-1}, four hours before inoculation. Subsequently, the seeds were placed in plastic bags and the inoculants were added using autoclaved pipettes. Then, the plastic bags were shaken manually until the seeds were completely covered.

Every experiment included two controls, non-inoculated control without N fertilizer (treatment 1), and non-inoculated control with N fertilizer (treatment 2). For soybean, 200 kg N ha^{-1} was applied as urea, being 50 % applied at 35 days after emergence. The inoculated controls were: single inoculation with *Bradyrhizobium* (treatment 3) and co-inoculation of *Bradyrhizobium* + *A. brasilense* AbV5/6 (treatment 4). The new strains were tested in co-inoculation with *Bradyrhizobium*, as follows: *Bradyrhizobium* + *A. brasilense* IH1 (treatment 5), *Bradyrhizobium* + *A. brasilense* HM053 (treatment 6), *Bradyrhizobium* + *A. brasilense* HM210 (treatment 7) and *Bradyrhizobium* + *A. brasilense* IH1 + HM053 + HM210 (treatment 8). The experiments were designed as completely randomized blocks with six replicates. To perform seed co-inoculation, the dose of *Bradyrhizobium* and the dose of *A. brasilense* were mixed in a sterile bottle for subsequent seeds inoculation (HUNGRIA; NOGUEIRA; ARAUJO, 2015).

1.2.2 Sites description and field management

Field experiments were performed in the following five cities: Mandaguaçu and Dois Vizinhos in Paraná state, Pindorama in São Paulo state and Rio Verde and Indiara in Goiás state. The characteristics of each location are shown in Table 11.

Table 1.1 – Characteristics of the experimental sites, type of soil, climate and precipitation during the conduction of the experiments.

Location	Coordination	Soil Type	Climate classification	Accumulated Precipitation*
Mandaguaçu	23º23’45” S 52º08’27” W, 507 m a.s.l	Oxisol (Latossolo Vermelho Distroférrico)	Subtropical (Cfa)	690 mm
Dois Vizinhos	25°69’40.1’’S 53°09’23.2’’ W, 524 m a.s.l	Oxisol (Latossolo Vermelho Distroférrico)	Subtropical (Cfa)	500 mm
Pindorama	21° 13' S 48° 55' W, 546 m a.s.l.	NI	Aw	766.4 mm
Rio Verde	17°47'53" S 50°55'41" W, 742 m a.s.l.	Oxisol (Latossolo Vermelho)	Aw	1225 mm
Indiara	17° 11' 04" S 49° 59' 04" W, 572 m a.s.l.	Oxisol (Latossolo amarelo)	Aw	1390 mm

* Accumulated Precipitation during the entire experiment.

For the two experiments carried out at Goiás, the experimental plots measured 6.0 m (length) × 4.0 m (width) and were separated by 0.5 m rows, with a population of 440,000 plants per hectare. At Dois Vizinhos, the plots measured 5.0 m (width) × 9.0 m (length), and the line spacing was 0.45 m, totaling 266,666 plants per hectare. At Pindorama, the plots measured 4.0 m (width) × 6.0 m (length), and the line spacing was 0.5 m, totaling 300,000 plants per hectare. Fungicides and insecticides were used in all treatments and none of the experiments was irrigated.

Before sowing, soil samples were randomly taken at each site, from the 0-10 or 0-20 cm layer. It was dried (60 °C for 48 h) and sieved (2.00 mm) for chemical and physical characterization. The main chemical and physical soil properties for all the five locals are shown in Table S1.

The base fertilization varied in each location, as well as the soybean cultivar, as each region has different soil and climate conditions, therefore, the most adapted cultivar in each region was chosen. Detailed information on base fertilization and cultivars used are shown in Table 1.2.

Table 1.2: Basal fertilization, cultivar and predecessor culture in the different places where the experiment was carried out.

Site	Base fertilization		Cultivar	Predecessor culture	Harvested area
	Formulation	**Dose (kg ha^{-1})**			**m²**
Mandaguaçu	04-30-10	250	M 6410 IPRO	NI*	9
Dois Vizinhos	00-18-18	500	BRS 511	NI*	3,6
Pindorama	00-20-20	300	TMG 7062 IPRO	Pasture	12
Rio Verde	00-30-10	400	Bônus	Maize	16
Indiara	00-30-10	400	Bônus	Maize	16

*NI-Not informed.

In all experiments and treatments, two foliar fertilization applications with 150 ml ha^{-1} of cobalt (3.5 g of Co) and molybdenum (35 g of Mo) was carried; the first application being between the phenological stages V3 and V4 and the second at R1.

1.2.3 Plant sampling and harvesting

Nodulation (number of nodules and dry mass of nodules) was evaluated 35 days after emergence in the cities of Rio Verde and Indiara (GO) and at flowering in the cities of Dois Vizinhos, Pindorama and Mandaguaçu. Five plants were collected from each experimental plot (avoiding central rows established for grain yields). In the laboratory, shoots were separated from roots and the latter was carefully washed and placed in an air-forced dryer at 65 °C until achieve constant weight (approximately 72 h). Nodules were removed from roots and dried again, for determination of nodulation (nodule number and dry weight). Shoot dry weight was evaluated, as well as shoot N content by the Kjeldahl digestion method (MALAVOLTA; VITTI; OLIVEIRA, 1997).

Grain yield at physiological maturity was determined by harvesting the central area of each plot, the harvested areas are shown in Table 1.2 Grains were cleaned, weighed and the values were corrected to 13 % moisture content, after the determination of humidity in a grain moisture tester. An evaluation of the N content in the grains was also carried out, with the same methodology mentioned above.

1.2.4 Statistical analyses

The data obtained in the Dois Vizinhos, Pindorama, Indiara and Rio Verde experiments were submitted to normality test, homocedasticity of variances and analysis of variance (ANOVA), and when significance at 5% was found, the differences were compared by Tukey's test at 5 %. At Mandaguaçu, were compared means using the LSD test, at the same level of significance.

1.3 RESULTS

1.3.1 Effect of co-inoculation of new *A. brasilense* strains on nodulation and growth parameters of soybean plants

At Goiás, the experiment was carried out in two experimental areas and all parameters showed statistical difference among treatments. For the total number of nodules (NN), at Rio Verde, all co-inoculations, apart from strain HM210, were statistically superior to the single inoculation with *Bradyrhizobium* and all treatments that received inoculation or co-inoculation showed a greater number of nodules compared to control and mineral fertilization. Co-inoculation with IH1 increase 26 nodules in relation to control treatment and 36 nodules in relation to the fertilized treatment. At Indiara, the results were similar, all new strains of *A. brasilense* were statistically equal to co-inoculation with AbV5/6.

Table 1.3: Total nodule number (NN), total nodule dry weight (NDW), shoot dry mass (SDW) and total shoot nitrogen content (TSNC) of soybean plants collected 35 days after emergence – Rio Verde - GO – 2019/20.

	Treatment	**NN**		**NDW**		**SDW**		**TSNC**	
		nº plant^{-1}		**mg plant^{-1}**		**g plant^{-1}**		**mg kg^{-1}**	
1	Non-inoculated control	15.1	d	25.5	c	11.8	c	26.4	c
2	Fertilization with mineral N	5.8	e	12.3	d	23.3	a	36.7	a
3	Brady	32.8	c	46.5	b	21.1	b	35.2	ab
4	Brady + Azo AbV5/6	41.5	ab	57.3	a	21.6	ab	34.3	b
5	Brady + Azo IH1	41.8	a	48.6	ab	21.3	b	35.8	ab
6	Brady + Azo HM053	38.5	ab	52.6	ab	21.5	ab	35.1	ab
7	Brady + Azo HM210	36.1	bc	51.5	ab	21	b	35.4	ab
8	Brady + Azo (IHI + HM053 + HM210)	39.0	ab	54.3	ab	21.1	b	35.0	ab

Averages followed by the same letter in the column do not statistically differ from each other by Tukey test ($p \leq 0.05$).

Regarding nodules dry weight (NDW), at Indiara, all inoculated or co-inoculated treatments were superior to the control treatment and mineral fertilization. At Rio Verde, co-inoculation with AbV5/6 was superior to single inoculation (treatment 3). All other co-inoculation combinations with the new *A. brasilense* strains did not differ from the co-inoculated treatment with AbV5/6 and the single inoculation with *Bradyrhizobium*.

For shoot dry weight (SDW), at Rio Verde, the treatments co-inoculated with AbV5/6 and HM053 were statistically equal to the treatment that only received mineral fertilizer, being superior to control, showing that the bacteria have the potential to provide nitrogen for plants to develop efficiently. At Indiara, the fertilized treatment and all other treatments were superior to control, without inoculation and without fertilization.

Table 1.4: Total nodule number (NN), total nodule dry weight (NDW), shoot dry mass (SDW) and total shoot nitrogen content (TSNC) of soybean plants collected 35 days after emergence – Indiara - GO – 2019/20.

	Treatment	**NN**		**NDW**		**SDW**		**TSNC**	
		nº plant^{-1}		**mg plant^{-1}**		**g plant^{-1}**		**mg kg^{-1}**	
1	Non-inoculated control	19.1	d	35.5	b	13.3	b	25.7	b
2	Fertilization with mineral N	9.3	e	19.8	c	25.6	a	32.3	a
3	Brady	48.6	c	56.5	a	23.0	a	33.9	a
4	Brady + Azo AbV5/6	61.5	a	59.0	a	24.5	a	34.1	a
5	Brady + Azo IH1	59.8	ab	59.1	a	25.1	a	34.7	a
6	Brady + Azo HM053	56.0	ab	59.8	a	25.1	a	34.7	a
7	Brady + Azo HM210	57.1	ab	58.5	a	25.3	a	35.1	a
8	Brady + Azo (IHI + HM053 + HM210)	53.3	bc	58.6	a	24.3	a	34.9	a

Averages followed by the same letter in the column do not statistically differ from each other by Tukey test ($p \leq 0.05$).

For the N content in shoot (TSNC), at Rio Verde, all the co-inoculations with the new strains were statistically equal to the treatment that received nitrogen fertilization and co-inoculation with AbV5/6. However, the AbV5/6 strain was inferior to the treatment that received only fertilization. At Indiara, all treatments that received inoculation or co-inoculation were equal to the fertilized treatment and superior to control.

In the experiment carried out at Pindorama (SP), both the single inoculation with *Bradyrhizobium* and the co-inoculations with strains of *A. brasilense* showed statistical superiority for the number of total nodules in relation to the control treatment and the treatment that received only mineral fertilizer. However, the best performance in the number of nodules was with the co-inoculation of *A. brasilense* AbV5/6, with 31 nodules per plant. Strains IH1, HM053 and the mixed co-inoculation were statistically equal to the single inoculation with *Bradyrhizobium*. For total nodule dry weight, the best results were found with single inoculation and co-inoculation with AbV5/6. The co-inoculation with the new strains of *A. brasilense* were statistically superior to control and treatment with mineral fertilizer.

For SDW, treatments with mineral fertilizer and mixed co-inoculation (treatment 8) showed the highest values. Co-inoculation with the new strains of *A. brasilense* (treatments 5, 6 and 7) were statistically equal to co-inoculation with AbV5/6, showing that they are capable of benefiting plant development. For the nitrogen content in the shoot of the plants, all treatments that received co-inoculation with *A. brasilense* were statistically equal to the treatment that received only mineral fertilization. It shows that all strains of *A. brasilense* tested in this study showed to supply together with *Bradyrhizobium* the nitrogen demand of soybean plants since all treatments with *A. brasilense* presented equal performance in relation to mineral fertilization. This result justifies the fact that soybean crop does not require mineral nitrogen fertilization, as long as the seeds are inoculated with efficient bacteria.

Table 1.5 - Total nodule number (NN), total nodule dry weight (NDW), shoot dry weight (SDW) and total shoot nitrogen content (TSNC) of soybean plants collected at the phenological stage R1 – Pindorama - SP - 2019/20.

	Treatment	**NN**		**NDW**		**SDW**		**TSNC**	
		nº plant^{-1}		**mg plant^{-1}**		**g plant^{-1}**		**g kg^{-1}**	
1	Non-inoculated control	4,77	de	46,33	d	11,40	c	23,07	b
2	Fertilization with mineral N	2,53	e	28,1	e	21,53	a	29,55	a
3	Brady	15,83	b	296,77	a	13,00	c	23,39	b
4	Brady + Azo AbV5/6	31,87	a	314,3	a	14,27	bc	28,08	a
5	Brady + Azo IH1	12,37	bc	131,27	c	13,80	bc	27,15	a
6	Brady + Azo HM053	13,17	bc	205,4	b	11,67	c	28,99	a
7	Brady + Azo HM210	8,37	cd	177,7	bc	12,40	c	28,69	a
8	Brady + Azo (IHI + HM053 + HM210)	15,3	b	189,77	b	18,60	ab	29,64	a

Averages followed by the same letter in the column do not statistically differ from each other by Tukey test ($p \leq 0.05$).

At Mandaguaçu, all treatments that received co-inoculation with the new strains of *A. brasilense* showed statistically higher values than controls without inoculation (treatments 1 and 2), single inoculation with *Bradyrhizobium* (treatment 3) and standard co-inoculation with *A. brasilense* AbV5/6 (treatment 4), for the number of nodules and for the dry weight of nodules (Table 1.6). The treatment that stood out the most was with the strain *A. brasilense* HM053, which presented 59 more nodules when compared to co-inoculation with AbV5/6 and more than 90% increase in the dry weight of nodules.

Table 1.6 - Total nodule number (NN), total nodule dry weight (NDW), shoot dry weight (SDW) and total shoot nitrogen content (TSNC) of soybean plants collected at the phenological stage R1 – Mandaguaçu - PR - 2019/20.

	Treatment	NN		NDW		SDW		TSNC	
		nº plant^{-1}		**mg plant^{-1}**		**g/?**		**g kg^{-1}**	
1	Non-inoculated control	61,53	f	158,85	ef	10,23	g	33,06	h
2	Fertilization with mineral N	43,75	g	132,1	f	14,34	f	48,78	g
3	Brady	79,33	e	202,46	e	16,74	ef	52,27	f
4	Brady + Azo AbV5/6	100	d	262,9	d	18,71	de	54,94	e
5	Brady + Azo IH1	135,86	b	413,84	b	26,05	b	61,55	b
6	Brady + Azo HM053	159,42	a	515,24	a	36,76	a	64,99	a
7	Brady + Azo HM210	125,68	c	384,68	bc	23,57	bc	59,56	c
8	Brady + Azo (IHI + HM053 + HM210)	119,43	c	332,51	c	20,75	cd	57,1	d

Averages followed by the same letter in the column do not statistically differ from each other by LSD test ($p \leq 0.05$).

The result of the good development of the nodules with the co-inoculations is reflected in the parameters of plant development, since the treatment with HM053 was also superior to all other treatments for the variables SDW and TSNC. All new strains tested showed better results than the control treatments (treatments 1, 2, 3 and 4). For SDW, strain HM053 showed the highest increase, being more than 3 times higher than the absolute control.

At Dois Vizinhos (PR), the treatments that received co-inoculation with AbV5/6, IH1 and HM053 and the control treatment (without inoculation and without fertilization) were statistically equal to the single inoculation with *Bradyrhizobium*, and the co-inoculated treatments presented the highest values of NDW. These treatments, on average, showed an increase of 64.5% in relation to the treatment that received only mineral N fertilization. It is noteworthy that the treatment that received *Bradyrhizobium* + *A. brasilense* HM053 had the highest NN and NDW.

The co-inoculation with *Bradyrhizobium* + *A. brasilense* HM053 presented the highest SDW value (Table 1.7), being statistically equal to the treatment that received the co-inoculation with *A. brasilense* HM210, both treatments are statistically superior to the others. The SDW of the treatment with HM053 presented a 64% increase in relation to the standard inoculation, only with *Bradyrhizobium*.

Table 1.7 - Total nodule number (NN), total nodule dry weight (NDW), shoot dry weight (SDW) and total shoot nitrogen content (TSNC) of soybean plants collected at the phenological stage R1 – Dois Vizinhos - PR - 2019/20.

	Treatment	NN		NDW		SDW		TSNC	
		nº plant^{-1}		**mg plant^{-1}**		**g plant^{-1}**		**g kg^{-1}**	
1	Non-inoculated control	66,56	bc	196,96	ab	22,71	cd	25,07	ab
2	Fertilization with mineral N	68,90	abc	79,56	c	24,69	cd	25,09	ab
3	Brady	63,23	c	216,33	a	19,85	d	24,56	ab
4	Brady + Azo AbV5/6	63,40	c	222,96	a	26,92	bc	22,50	b
5	Brady + Azo IH1	73,26	ab	225,63	a	23,49	cd	24,45	ab
6	Brady + Azo HM053	75,93	a	231,56	a	32,71	a	26,11	a
7	Brady + Azo HM210	62,10	c	156,50	b	31,31	ab	25,23	a
8	Brady + Azo (IHI + HM053 + HM210)	52,43	d	154,66	b	22,97	cd	24,52	ab

Averages followed by the same letter in the column do not statistically differ from each other by Tukey test ($p \leq 0.05$).

For the TSNC, the co-inoculation with *Bradyrhizobium* + *A. brasilense* HM053 and *Bradyrhizobium* + *A. brasilense* HM210 were statistically superior to the standard co-inoculation with *Bradyrhizobium* + *A. brasilense* AbV5/AbV6, with an increase of 16% and 12% respectively.

1.3.2 Effect of co-inoculation with strains of *A. brasilense* on soybean yield

At Rio Verde and Indiara (GO), the control and the treatment that received only mineral fertilizer (treatment 2), were statistically inferior to all other treatments, presenting the lowest productivity, on average, for the control of 2464 kg ha^{-1} and 2466 kg ha^{-1} for the fertilized treatment. In the experiment carried out at Indiara, all treatments that received co-inoculation with any of the *A. brasilense* strains were statistically superior to the treatment that received only inoculation with *Bradyrhizobium*, and all new strains matched the performance of the AbV5/6. However, at Rio Verde, the treatments co-inoculated with AbV5/6 and HM210 were statistically superior to the single inoculation with *Bradyrhizobium*, however, the treatments co-inoculated with IH1, HM053 and the mixture of the three new strains are equal to the performance of AbV5/6 and HM210.

Table 1.8 – Nitrogen content in grains, total nitrogen in grains and soybean grain yield – Rio Verde - GO – 2019/20.

Treatment		N grain mg kg^{-1}		N total kg ha^{-1}		Yield kg ha^{-1}	
1	Non-inoculated control	36,2	c	84,5	c	2332	c
2	Fertilization with mineral N	32,4	c	80,8	c	2501	c
3	Brady	65,9	b	234,7	b	3551	b
4	Brady + Azo AbV5/6	70,5	a	268	a	3816	a
5	Brady + Azo IH1	67,8	ab	252,8	ab	3719	ab
6	Brady + Azo HM053	65,3	b	240,6	b	3703	ab
7	Brady + Azo HM210	66,7	ab	254,4	ab	3810	a
8	Brady + Azo (IHI + HM053 + HM210)	67,1	ab	248,3	ab	3708	ab

Averages followed by the same letter in the column do not statistically differ from each other by Tukey test ($p \leq 0.05$).

Analyzing the N content in the grains and the total N value in the grains at Indiaria, it is noted that all treatments that received inoculation/co-inoculation were statistically equal to each other and superior to the control and the fertilized treatment (treatment 2). Therefore, at Rio Verde, for the N content in the grains and total N in the grains, the co-inoculation with AbV5/6 was statistically superior to the control, the fertilized treatment (treatment 2), the single inoculation with *Bradyrhizobium* and the co-inoculation inoculation with HM053.

Table 1.9 – Nitrogen content in grains, total nitrogen in grains and soybean grain yield – Indiara - GO – 2019/20.

Treatment		N grain mg kg^{-1}		N total kg ha^{-1}		Yield kg ha^{-1}	
1	Non-inoculated control	37,1	b	96,3	c	2596	c
2	Fertilization with mineral N	37,6	b	91,8	c	2431	c
3	Brady	66,2	a	244	b	3683	b
4	Brady + Azo AbV5/6	70,7	a	292,9	a	4140	a
5	Brady + Azo IH1	68,5	a	281	a	4101	a
6	Brady + Azo HM053	68,9	a	279,8	a	4063	a
7	Brady + Azo HM210	69,3	a	278,5	a	4014	a
8	Brady + Azo (IHI + HM053 + HM210)	69,0	a	274,7	a	3708	ab

Averages followed by the same letter in the column do not statistically differ from each other by Tukey test ($p \leq 0.05$).

At Pindorama (SP), for grain yield, all treatments that received inoculation or co-inoculation were statistically superior to the control treatment (treatment 1). Regarding the new strains tested, all combinations showed the same performance as standard co-inoculation with *A. brasilense* AbV5/6, being efficient in promoting plant growth, resulting in significant increases in grain productivity. The highest productivity was obtained with the co-inoculation of *A. brasilense* HM053, which was 4686.90 kg ha^{-1}.

Table 1.10 – Nitrogen content in grains and soybean grain yield – Pindorama - SP – 2019/20.

	Treatment	**N grain g kg^{-1}**		**Yield kg ha^{-1}**	
1	Non-inoculated control	57,9	d	3264,3	d
2	Fertilization with mineral N	66,05	ab	4068,6	bc
3	Brady	65,46	cd	4008,1	c
4	Brady + Azo AbV5/6	65,57	abc	4338,4	abc
5	Brady + Azo IH1	62,76	bc	4126,6	bc
6	Brady + Azo HM053	67,69	a	4686,9	a
7	Brady + Azo HM210	62,16	bc	4518,7	ab
8	Brady + Azo (IHI + HM053 + HM210)	64,5	abc	4398,4	abc

Averages followed by the same letter in the column do not statistically differ from each other by Tukey test ($p \leq 0.05$).

The nitrogen content in the grains showed statistical differences among treatments. The co-inoculation with HM053 presented the highest average, with 67.69 g kg^{-1}. Treatments with mineral fertilizer, co-inoculation with AbV5/6 and mixed co-inoculation were statistically equal to co-inoculation with HM053. Here again, it is possible to demonstrate that the bacteria are able to supply an amount of nitrogen equal to those plants that received mineral fertilizer, with the advantage of being a more sustainable option for the production system and the environment.

At Mandaguaçu, for the productivity parameters, there was the same behavior found for nodulation in the topic above; the new strains tested showed superior results to the other treatments, presenting on average an increase of 9% in nitrogen content in grains, 22% in total nitrogen in grains and 11.5% in grain yield in relation to the co-inoculation with AbV5/6. Strain HM053 obtained the best performance for these analyzed variables, obtaining a grain yield increase of 21.7% in relation to the co-inoculation with AbV5/6 and 25.4% in relation to the single inoculation with *Bradyrhizobium*.

Table 1.11: Nitrogen content in grains, total nitrogen in grains and soybean grain yield – Mandaguaçu - PR – 2019/20.

	Treatment	N grain g kg^{-1}		N total kg ha^{-1}		Yield kg ha^{-1}	
1	Non-inoculated control	45.97	g	142.15	h	3091.96	g
2	Fertilization with mineral N	50.23	f	167.10	g	3326.79	f
3	Brady	56.27	e	194.36	f	3454.03	e
4	Brady + Azo AbV5/6	56.75	e	201.93	e	3558.29	e
5	Brady + Azo IH1	62.71	b	251.35	b	4008.16	b
6	Brady + Azo HM053	65.12	a	282.08	a	4332.11	a
7	Brady + Azo HM210	61.66	c	237.51	c	3851.89	c
8	Brady + Azo (IHI + HM053 + HM210)	58.70	d	215.76	d	3677.52	d

Averages followed by the same letter in the column do not statistically differ from each other by LSD test (p≤0.05).

The single inoculation with *Bradyrhizobium* was the highlight in grain yield at Dois Vizinhos, being superior to all other treatments. For the co-inoculated treatments, the best performance was for the AbV5/6 strain, not statistically different from the control treatment. Then, mixed co-inoculation (treatment 8) was statistically equal to co-inoculation with HM053. The treatment that only received mineral N fertilization (treatment 2) had the lowest grain yield (2238.52 kg ha^{-1}), and this result may be associated with the low values of NDW and SDW found in this treatment.

Table 1.12: Nitrogen content in grains, total nitrogen in grains and soybean grain yield – Dois Vizinhos - PR – 2019/20.

	Treatment	N grain g kg^{-1}		N total kg ha^{-1}		Yield kg ha^{-1}	
1	Non-inoculated control	65.55	de	222.20	b	3721.97	b
2	Fertilization with mineral N	69.38	ab	209.19	c	2238.52	f
3	Brady	64.55	e	224.55	ab	4170.21	a
4	Brady + Azo AbV5/6	68.23	bc	236.19	a	3767.33	b
5	Brady + Azo IH1	66.04	de	216.56	bc	2500.03	ef
6	Brady + Azo HM053	66.77	cd	191.95	d	2905.29	cd
7	Brady + Azo HM210	68.44	abc	193.73	d	2757.92	de
8	Brady + Azo (IHI + HM053 + HM210)	70.15	a	214.12	bc	3152.72	c

Averages followed by the same letter in the column do not statistically differ from each other by Tukey test (p≤0.05).

The mixed co-inoculation (treatment 8) showed 70.15 g kg^{-1} of nitrogen in the soybean grains, the highest average between the treatments, not statistically different from the treatment that received mineral fertilization and the co-inoculation with the HM210 strain. Comparing the mixed co-inoculation with the single inoculation with *Bradyrhizobium* (treatment 3), there was an increase of 8.6% and 2.8% in relation to the standard co-inoculation with the AbV5/6 strain.

For the total nitrogen in the grains, the co-inoculation of *Bradyrhizobium* + *A. brasilense* AbV5/AbV6 was statistically superior to the other treatments, not differing only from the single inoculation with *Bradyrhizobium*. The co-inoculation provided an increase in total N in the grains of 6.2% when compared to the control treatment (treatment 1) and 12.9% when compared to the treatment that received nitrogen fertilization (treatment 2).

1.4 DISCUSSION

Based on a possible increase of plant nodulation in response to changes caused by *Azospirillum* in root morphology (SAIKIA et al. 2010), nodule parameters were evaluated 35 days after emergence and at flowering. Nodulation was evaluated in these phenological studies, since there are reports that co-inoculation with *A. brasilense* in soybean promotes early nodulation in soybean plants (CHIBEBA et al. 2015) and according to Barbosa et al. (2021) there is a positive and significant relationship between nodulation parameters (number and weight) and grain yield.

Diverse responses were detected in the different tested places. At 35 days after plant emergence, at Rio Verde, co-inoculation with AbV5/6, HM053, IH1 and the mix of strains generated statistically equal numbers of nodules, which were higher than the numbers of nodules obtained after a single inoculation with *Bradyrhizobium*. Regarding the weight of nodules, both inoculation and co-inoculations were efficient in increasing this parameter since they were statistically superior to control without inoculation and to the treatment that received only mineral fertilizer.

For SDW at 35 days after plant emergence, the HM053 strain stood out at Rio Verde, where it was statistically equal to the co-inoculation with AbV5/6 and to the treatment that received only mineral fertilization. In the literature, there are no responses to the inoculation of the HM053 strain in soybeans, however, Santos et al. (2017a) evaluated this strain in wheat. HM053 strain was able to colonize the surface of the roots and to increase the dry weight of the shoot and root by 30 and 49%, respectively, when compared to non-inoculated plants and by 30 and 31% when compared to the parent strain FP2.

During the flowering stage, at Mandaguaçu, strain HM053 stood out, which increased NN, NDW, SDW and TSNC, being statistically superior to all other treatments. Studies have already demonstrated the efficiency of the HM053 strain in promoting plant growth and fixing nitrogen. *Setaria viridis* inoculated with the HM053 strain incorporated a significant level of N via biological fixation and could obtain enough nitrogen to supply the daily demand of N and promote its growth (PANKIEVICZ et al., 2015). The explanation for the good performance of this strain, according to Machado et al. (1991), is the ability to excrete ammonium and is related to the low activity of glutamine synthetase, resulting in a deficiency in the assimilation of NH_4^+, which justifies the excretion of excess ammonium produced during the process of nitrogen fixation. Recent studies show that inoculation of maize seeds with the HM053 strain increased plant iron assimilation and translocation to the aerial portions during early stages of development, resulting in significant increases in crop yield and iron content in the seeds (HOUSH et al., 2021). Strain HM053 is known to produce high levels of auxins and can promote plant growth by improving host manganese assimilation, which in turn improves the photosynthetic capacity of plants (HOUSH et al., 2022).

The N content of the shoot during the flowering period at Pindorama demonstrated the efficiency of all strains of *A. brasilense* in performing BNF, since all co-inoculations were superior to the single inoculation of *Bradyrhizobium* and equal to the treatment that received mineral fertilization, demonstrating the great potential of these bacteria to supply nitrogen to plants. Through a meta-analysis study, it was possible to observe that the co-inoculation of soybean with *Azospirillum* provided a significant increase of 11.05% in the number of nodules, 14.65% in the biomass of nodules and 6.39% in the biomass of aerial part (ZEFFA et al., 2020). In all places where NDW was evaluated in the flowering of soybean plants, it was evident that mineral fertilization impairs the formation of nodules. Research has shown that mineral N, applied in the sowing furrow, can reduce nodulation and the efficiency of BNF, in doses greater than 20 kg ha^{-1} (HUNGRIA and VARGAS, 2000). In this experiment, the dose of 200 kg ha^{-1} of N in the form of urea was divided into 100 kg ha^{-1} at sowing and 100 kg ha^{-1} at flowering, thus justifying the harmful effect of nitrogen fertilization on the NDW of the treatment 2.

At Dois Vizinhos, the HM053 strain again stood out in the nodulation parameters, where it presented the highest NN, being statistically superior to the co-inoculation with AbV5/6, with an increment of 19.7% and also presented the highest value of NDW. The good performance in nodulation may have reflected in better performances in the aerial part of the plants, as this strain was also responsible for the highest value of SDW and TSNC, generating a significant increase of 21.5% and 16% respectively, in relation to co- inoculation with AbV5/6. According to Chibeba et al. (2015), the increase in plant growth in response to *Azospirillum* can be attributed to the greater availability of N due to biological fixation and the presence of hormones that stimulate plant growth. These theorie, corroborates with the new hypothesis explaining the action mechanism of *Azospirillum* proposed by Cassán et al. (2020), called the efficient nutrient acquisition hypothesis, which postulates that the promotion of plant growth occurs through two main mechanisms, biological N fixation and phytohormone production, which are effectively induced by colonized bacteria.

Analyzing grain productivity, the new strains of *A. brasilense* showed equal or greater yields than that achieved with AbV5/6. In the experiment carried out at Mandaguaçu, all the new strains were statistically superior to the controls and the standard co-inoculation with AbV5/6, with emphasis on the HM053 strain, which reached 21.7% increment in relation to the co-inoculation with AbV5/ 6 and 25.4% in relation to the single inoculation with *Bradyrhizobium*. At Pindorama, all co-inoculations with the new strains were statistically equal to the co-inoculation with AbV5/6 and superior to the control, and, again, the highest productivity was found with the HM053 strain. Pedrosa et al. (2019) found promising results with the inoculation of *A. brasilense* HM053 in maize, where its use almost doubled the increase in productivity provided by the commercial strain, *A. brasilense* AbV5.

According to Barbosa et al. (2021), soybean cultivars with determinate growth habit respond better to co-inoculation. In this work, all cultivars used had an indeterminate habit, with the exception of Pindorama, where the growth habit was semi-determinate. Therefore, the variability of results among cultivars and bacterial interaction may be related to the intrinsic genetic characteristics of each soybean genotype (NAOE et al., 2020).

The experiment carried out at Dois Vizinhos showed the lowest grain yield average, when compared to the other locations, and the highest grain yield was found with the single inoculation of *Bradyrhizobium*. Among the co-inoculations, AbV5/6 presented the highest average, followed by mixed inoculation (treatment 8) and strain HM053. This experiment was affected by a water deficit at the beginning of the crop cycle, which may have influenced bacterial colonization and seedling establishment. Hungria, Nogueira and Araujo (2015) reported that water and temperature stress are among the most limiting factors for biological nitrogen fixation in the tropics, seriously compromising root infection, nodule formation and N fixation. Analysis report that under water stress conditions, co-inoculation was not effective in increasing soybean productivity (BARBOSA et al., 2021). However, even if inoculation does not result in higher grain yields, this technique is the most sustainable way to supply N to soybeans and ensure that, among the various factors that comprise production components, at least N is the least restrictive possible (CHALK et al., 2010). Despite the co-inoculations not having obtained good results in the grain yield at Dois Vizinhos, this was not reflected in the N uptake by the plants, since the mixed co-inoculation (treatment 8) presented the highest average for the N content in the grains soybean, being higher than that found with the AbV5/6 strain.

At Rio Verde and Indiara, the new strains had similar yields, since the co-inoculation of the new strains induced performance equal to AbV5/6, or equal or greater than that found with the single inoculation with *Bradyrhizobium*, reinforcing the potential that these strains have in promoting plant growth and in most cases, generating an increase in grain yield. The effects generated by the use of *A. brasilense* are explained through several mechanisms. Studies report that *A. brasilense* increases root hair length in response to cell differentiation and elongation triggered by auxins and ethylene produced by the bacteria (PITTS et al., 1998; VACHERON et al., 2013). As a result, the abundance of root hairs and their elongation promote a more effective contact between the roots and the soil, increasing the plant's potential to absorb water and nutrients (HALING et al., 2013). In addition, other molecules synthesized by *A. brasilense*, such as salicylic acid and jasmonate, can induce plant tolerance to oxidative stress (FUKAMI et al., 2018).

The new strains of *A. brasilense* were also efficient in increasing the amount of nitrogen in the grains in most places. Strain HM053 promoted higher N content in grains in the cities of Pindorama, Mandaguaçu and Dois Vizinhos, with increments of 3.4% in Pindorama and Dois Vizinhos and 15.7% in Mandaguaçu. Galindo et al. (2022) found similar results for cowpea: 13% of increment in grain N content due to the co-inoculation with *A. brasilense* when compared to single inoculation with *Bradyrhizobium*. According to Benintende et al. (2010), co-inoculation of soybeans with *Azospirillum* and *Bradyrhizobium* increased N_2 fixation and grain yield. In addition, Mathukumar and Udaiyan (2018) claim that the co-inoculation of microorganisms with different functions can be considered an economically viable and environmentally sustainable strategy to improve plant performance.

A study carried out by Lopes et al. (2022) on lima bean, evaluating the co-inoculation of *A. baldaniorum* and *Bradyrhizobium*, suggest that co-inoculation has a significant effect on nodulation and N fixation. Lopes et al. (2022) believe that *A. baldaniorum* helped the rhizobia to perform BNF properly, even when exposed to salinity, due to the release of phytohormones, mainly AIA, ABA, cytokinin, gibberellin and salicylic acid.

The results of a meta-analysis demonstrated the lack of a positive and significant contribution of co-inoculation to soybean grain yield, however, indirect evidence reports that the identification of bacterial strains that cause complementary effects on plant development is a crucial step for the development of more efficient inoculants for the soybean crop (ZEFFA et al., 2020). A summary of the general average of grain yield of all evaluated sites and treatments (Figure 1.2a) shows increments of the association of *Bradyrhizobium* and *A. brasilense* in relation to the control and the treatment that received only mineral fertilization. The highest yields were found with treatments co-inoculated with HM053 and AbV5/6, with yields of 3938.06 kg ha^{-1} and 3924.00 kg ha^{-1}, respectively, resulting in increments in the order of 31.2% and 30.7% in relation to the control (treatment 1). *A. brasilense* HM053 proved to be a new alternative for co-inoculation in soybean, since it achieved greater productivity than the commercial strain AbV5/6 (Figure 1.2b).

When comparing the productivity means among the treatment that received only inoculation with *Bradyrhizobium* and the treatment that received co-inoculation with *A. brasilense* HM053, it is noted that in the cities of Mandaguaçu, Pindorama and Indiara, co-inoculation with HM053 was statistically superior the single inoculation (table 1.11, 1.10 and 1.9, respectively), providing increments of 878, 678 and 380 kg/ha respectively. Considering the current price of soybean in Brazil (May/23), these increases would result in gains of US$380, US$294 and US$165/ha, respectively, in these locations.

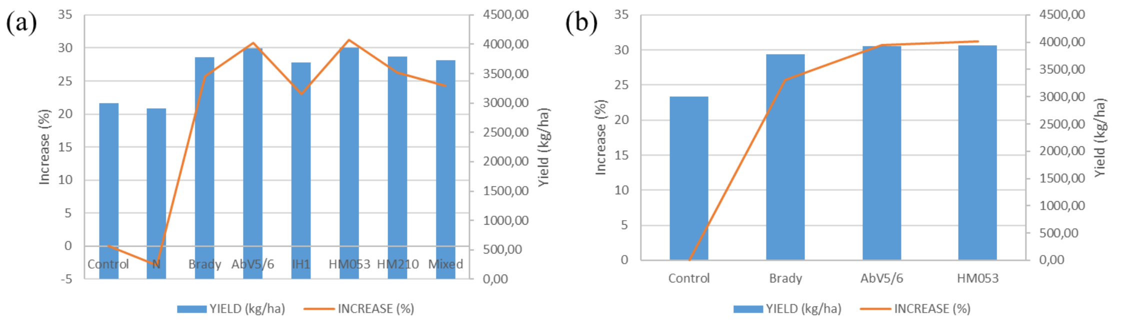

Figure 1.2: Effects of *A. brasilense* co-inoculation on the grain yield of soybean. Data represent the means values of all field experiments performed at Rio Verde, Indiara, Pindorama, Mandaguaçu and Dois Vizinhos. More details are given in the Table 1.8, 1.9, 1.10, 1.11 and 1.12.

Despite differences related to climatic and soil conditions of the study areas, the new strains of *A. brasilense* showed great potential, mainly the HM053 strain, which promoted an increase in all parameters evaluated in this study, with significant increases in soybean nodulation and grain yield, being a viable alternative for co-inoculation in soybean crop. The potential of HM053 to promote plant growth and improve productivity might be increased by developing inoculant formulations which guarantee longer bacteria viability under field conditions.

REFERENCES

ALI, T. et al. The Impact of Climate Change on China and Brazil's Soybean Trade. **Land**, v. 11, p. 2286, 2022.

ANPII, 2023. **National Association of Producers and Importers of Inoculants**. Available in: http://www.anpii.org.br/estatisticas/. Accessed 14.07.2023.

BARBOSA, J. Z. et al. Meta-analysis reveals benefits of co-inoculation of soybean with *Azospirillum brasilense* and *Bradyrhizobium* spp. in Brazil. **Applied Soil Ecology**, v. 163, 2021.

BASHAN, Y.; de-BASHAN, L. E. How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—a critical assessment. **Advances in Agronomy**, v. 108, p. 77–136, 2010.

BENINTENDE, S. et al. Comparación entre Coinoculación con Bradyrhizobium japonicum y *Azospirillum brasilense* e Inoculación simple con Bradyrhizobium japonicum en la Nodulación, Crecimiento y Acumulación de N en el Cultivo de Soja. **Agriscientia**, v. 27, p. 71-77, 2010.

BULEGON, L. G.; GUIMARÃES, V. F.; LAURETH, J. C. U. Azospirillum brasilense affects the antioxidant activity and leaf pigment content of Urochloa ruziziensis under water stress. **Pesq. Agropec. Trop.**, v. 46, p. 343-349, 2016.

CASSÁN, F. et al. Everything you must know about Azospirillum and its impact on agriculture and beyond. **Biology and Fertility of Soils**, v. 56, p. 461-479, 2020.

CASSÁN, F. et al. What Do We Know About the Publications Related with *Azospirillum*? A Metadata Analysis. **Microbial Ecology**, v. 81, p. 278-281, 2021.

CHALK, P. M. et al. Integrated effects of abiotic stresses on inoculant performance, legume growth and symbiotic dependence estimated by 15N dilution. **Plant Soil**, v. 328, p. 1–16, 2010.

CHIBEBA, A. M. et al. Co-Inoculation of Soybean with Bradyrhizobium and Azospirillum Promotes Early Nodulation. **American Journal of Plant Sciences**, v. 6, p. 1641-1649, 2015.

CHIBEBA, A. M. et al. Towards sustainable yield improvement: field inoculation of soybean with Bradyrhizobium and co-inoculation with Azospirillum in Mozambique. **Archives of Microbiology**, v. 202, p. 2579–2590, 2020.

DÍAZ-ZORITA, M.; FERNÁNDEZ-CANIGIA, M. V. Field performance of a liquid formulation of *Azospirillum brasilense* on dryland, wheat productivity. **Eur J Soil Biol**, v. 45, p. 3–11, 2009.

FERRI, G. C. et al. Effects of associated co-inoculation of *Bradyrhizobium japonicum* with *Azosprillum brasilense* on soybean yield and growth. **African Journal of Agricultural Research**, v. 12. p. 6-11, 2017.

FUKAMI, J., CEREZINI, P., HUNGRIA, M. *Azospirillum:* benefits that go far beyond biological nitrogen fixation. **AMB Express**, v. 8, p. 73, 2018.

GALINDO, F. S. et al. Technical and economic viability of co-inoculation with *Azospirillum brasilense* in soybean cultivars in the Cerrado. **Rev Bras Eng Agric Ambient**., v. 22, p. 51-56, 2018.

GALINDO, F. S. et al. Co-Inoculation with Azospirillum brasilense and Bradyrhizobium sp. Enhances Nitrogen Uptake and Yield in Field-Grown Cowpea and Did Not Change N-Fertilizer Recovery. **Plants**, v. 11, 2022.

GARCIA, M. V. C.; NOGUEIRA, M. A.; HUNGRIA, M. Combining microorganisms in inoculants is agronomically important but industrially challenging: case study of a composite inoculant containing *Bradyrhizobium* and *Azospirillum* for the soybean crop. **AMB Express**, v. 11, 2021.

HALING, R. E. et al. Root hairs improve root penetration, root-soil contact, and phosphorus acquisition in soils of different strength. **J Exp Bot**, v. 64, p. 3711–3721, 2013.

HIGUTI, I. H.; PEDROSA, F. O. (1985). Mutant of *Azospirillum brasilense* Sp7 that overproduces catalase. In: EVANS, H. J.; BOTTOMLEY, P. J.; NEWTON, W. E. (eds). **Nitrogen Fixation Research Progress**, Springer Netherlands, p. 532.

HOUSH, A. B. et al. Functional mutants of *Azospirillum brasilense* elicit beneficial physiological and metabolic responses in Zea mays contributing to increased host iron assimilation. **ISME J.,** v. 15, p. 1505-1522, 2021.

HOUSH, A. B. et al. *Azospirillum brasilense* Bacteria Promotes Mn2+ Uptake in Maize with Benefits to Leaf Photosynthesis. **Microorganisms,** v. 10, p. 1290, 2022.

HUNGRIA, M.; VARGAS, M. A. T. Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. **Field Crops Research**, v. 65, p.151-164, 2000.

HUNGRIA, M.; NOGUEIRA, M. A.; ARAUJO, R. S. Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability. **Biology and Fertility of Soils**, v. 49, p. 791-801, 2013.

HUNGRIA, M.; NOGUEIRA, M. A.; ARAUJO, R. S. Soybean Seed Co-Inoculation with Bradyrhizobium spp. and Azospirillum brasilense: A New Biotechnological Tool to Improve Yield and Sustainability. **American Journal of Plant Sciences**, v. 6, p. 811-817, 2015.

LOPES, A. L. O. et al. Synergism of Bradyrhizobium and *Azospirillum baldaniorum* improves growth and symbiotic performance in lima bean under salinity by positive modulations in leaf nitrogen compounds. **Applied Soil Ecology**, v. 180, 104603, 2022.

MACHADO, H. B. et al. Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine. **Can J Microbiol**, v. 37, p. 549–553, 1991.

MALAVOLTA, E.; VITTI, G. C.; OLIVEIRA, S. A. (1997). **Avaliação do estado nutricional das plantas: Princípios e aplicações.** Piracicaba, São Paulo, Brazil: Potafos.

MUTHUKUMAR, T.; UDAIYAN, K. Coinoculation of bioinoculants improve Acacia auriculiformis seedling growth and quality in a tropical Alfisol soil. **Journal of Forestry Research**, v. 29, p. 663–673, 2018.

NAOE, A. M. L. et al. Co-inoculation with Azospirillum brasilense in soybean cultivars subjected to water deficit. **Rev. Bras. Eng. Agríc. Ambient**., v. 24, p. 89-94, 2020.

PANKIEVICZ, V. C. S. et al. Robust biological nitrogen fixation in a model grass-bacterial association. **Plant Journal**, v. 81, p. 907–919, 2015.

PEDROSA, F. O. et al. The ammonium excreting Azospirillum brasilense strain HM053: a new alternative inoculant for maize. **Plant and Soil**, v. 451, p. 45-56, 2019.

PITTS, R.J.; CERNAC, A.; ESTELLE, M. Auxin and ethylene promote root hair elongation in Arabidopsis. **Plant J**, v. 16, p. 553–560, 1998.

PRANDO, A. M. et al. **Coinoculação da soja com *Bradyrhizobium* e *Azospirillum* na safra 2018/2019 no Paraná**. Londrina: Embrapa Soja - Circular Técnica 156, 19 p., 2019.

PRANDO, A. M. et al. **Coinoculação da soja com *Bradyrhizobium* e *Azospirillum* na safra 2020/2021 no Paraná**. Londrina: Embrapa Soja - Circular Técnica 181, 24 p., 2022.

SAIKA et al. (2010). Role of *Azospirillum* in the Improvement of Legumes. In: KHAN, M.S.; MUSARRAT, J.; ZAIDI, A. (eds). **Microbes for Legume Improvement**. Springer, Vienna, p. 389–408.

SANTOS, K.F.D.N. et al. Wheat colonization by an *Azospirillum brasilense* ammonium-excreting strain reveals upregulation of nitrogenase and superior plant growth promotion. **Plant and Soil**, v. 415, p. 245-255, 2017a.

SANTOS, A. R. S. et al. Labeled *Azospirillum brasilense* wild type and excretion-ammonium strains in association with barley roots. **Plant Physiology and Biochemistry**, v. 118, p. 422-426, 2017b.

SANTOS, M. S.; NOGUEIRA, M. A.; HUNGRIA, M. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. **AMB Express**, v.9, p. 1-22, 2019.

SANTOS, M. S.; NOGUEIRA, M. A.; HUNGRIA, M. Outstanding impact of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 on the Brazilian agriculture: Lessons that farmers are receptive to adopt new microbial inoculants. **Rev. Bras. Cienc. Solo**, v. 45, 2021.

SOUSA, M. S. **Inoculação de microrganismos promotores de crescimento na cultura da beterraba.** 2022, 53 p. Dissertação (Magister Scientiae) – Universidade Estadual do Oeste do Paraná, Marechal Cândido Rondon, 2022.

TIMMERS, A. C. J. (2000). Infection of root hairs by rhizobia: infection thread development with emphasis on the microtubular cytoskeleton. In: RIDGE, R. W., EMONS, A. M. C. (eds). **Root hairs**. Springer, Tokyo, p. 223–239.

VACHERON, J. et al. Plant grow-promoting rhizobacteria and root system functioning. **Front Plant Sci**, v. 4, p. 356, 2013.

ZANDI, P.; SCHNUG, E. Reactive Oxygen Species, Antioxidant Responses and Implications from a Microbial Modulation Perspective. **Biology**, v. 11, p. 155, 2022.

ZEFFA, D. M. et al. Effects of plant growth-promoting rhizobacteria on co-inoculation with *Bradyrhizobium* in soybean crop: a meta-analysis of studies from 1987 to 2018. **PeerJ.**, v. 8, p. e7905, 2020.

CHAPTER 2 – *Azospirillum brasilense* AbV5/6 ENCAPSULATION IN DUAL-CROSSLINKED BEADS BASED ON CATIONIC STARCH

Abstract: The main challenge of agriculture is feeding the growing population and at the same time providing environmental sustainability. Using *Azospirillum brasilense* as a biofertilizer has proved to be a promising solution. However, its prevalence in soil has not been efficient due to biotic and abiotic stresses. Thus, to overcome this drawback, we encapsulated the *A. brasilense* AbV5 and AbV6 strains in a dual-crosslinked bead based on cationic starch. The starch was previously modified with ethylenediamine by an alkylation approach. Then, the beads were obtained by a dripping technique, crosslinking sodium tripolyphosphate with a blend containing starch, cationic starch, and chitosan. The AbV5/6 strains were encapsulated into the hydrogel beads by a swelling diffusion method followed by desiccation. Plants treated with encapsulated AbV5/6 cells showed an increase in the root length by 19 %, shoot fresh weight by 17 %, and the content of chlorophyll b by 71 %. The encapsulation of AbV5/6 strains showed to keep *A. brasilense* viability for at least 60 days and efficiency to promote maize growth.

Key Words: Hydrogel, Plant growth promoting bacteria, Biotechnology, Maize, Sustainable agriculture

2.1 INTRODUCTION

One of the great challenges of the 21st century will be to feed the growing world population and at the same time reduce the environmental impact and the costs of agricultural production (FOLEY et al., 2011). The overexploitation of arable lands using unsustainable techniques may temporarily solve food demand, but they have adverse environmental effects. For example, the excessive application of chemical fertilizers (CANFIELD et al., 2010) and the deforestation to create new arable lands (PASTOR et al., 2019) further contribute to greenhouse gas emissions and climate changes (RICHARDSON et al., 2012; SMITH et al., 2013). Hence, there is an urgent need to change to more sustainable agricultural production practices that focus on increasing crop productivity, even in resource-limited and challenging environments (SHAH et al., 2021).

The use of Plant Growth-Promoting Bacteria (PGPB) as biofertilizers has grown significantly. PGPB are prokaryotes capable of colonizing soil and plants, benefiting host plants through mechanisms such as: biological nitrogen fixation, production of phytohormones, nutrient solubilization and protection against pathogens (TIMMUSK et al., 2017). In this way, it is possible to reduce the use of fertilizers, pesticides, mitigate biotic and abiotic stresses and increase productivity (GROVER et al., 2021).

Among the PGPB, *Azospirillum* is one of the most studied genera, as it has the ability to associate with approximately 113 plant species, distributed in 35 botanical families, including 14 cereal species (PEREG et al., 2016) and being marketed as an inoculant in several countries, such as Argentina, Mexico, India, Italy, France, and Brazil (BASHAN; de-BASHAN, 2010). The positive results over decades of research have proven the benefits of inoculating *Azospirillum* spp. in maize and wheat productivity (DÍAZ-ZORITA et al., 2015; HUNGRIA et al., 2010; PEDROSA et al., 2020). In Brazil, *A. brasilense* AbV5 and AbV6 are the most used strains for the formulation of commercial inoculants. As an inoculant, *Azospirillum* is supplied in peat or in a liquid formulation. When introduced directly into the soil, these carriers do not protect the PGPB from the toxic effects of pesticides, competition with other microorganisms, predation by microfauna and environmental variation (PONGSILP; NIMNOI, 2020; TAKAHASHI et al., 2022; URREA-VALENCIA et al., 2021). Therefore, the development of technologies that aim to protect *A. brasilense* from biotic and abiotic stresses in the soil, meets the need to create a favorable environment for this PGPB, increasing its survival in the soil and allowing more significant gains in agricultural productivity.

In this context, polymer technology may significantly contribute to developing new carriers with unique properties. Polymeric materials have been used in the most diverse technological areas, from medicine and engineering to agriculture (DERAKHSHANKHAH et al., 2022; QAMAR et al., 2022; SUPARE; MAHANWAR, 2022). Among the most promising polymeric materials, hydrogels (HG) are considered a particular class of soft materials that have attracted great interest in recent decades (KHAN et al., 2022).

Hydrogels are polymeric materials cross-linked in three dimensions that can absorb and retain a large amount of water, saline solutions, or biological fluids (PELLÁ et al., 2020). Such structures, when swollen, may contain a fraction of water higher than 90 % in weight. For agricultural applications, the HGs need to meet some requirements: i) the material must be biodegradable, ii) not expensive, iii) non-toxic, and iv) by-products from degradation reaction cannot be harmful to the environment (MAITZ, 2015). Thus, aiming to follow such requirements, hydrogels from natural sources (e.g. polysaccharides, polypeptides, or polyesters) have attracted much attention (D’AYALA et al., 2008). The biological environment recognizes such polymers facilitating their assimilation through metabolic degradation pathways. The research on hydrogels for agricultural applications has grown significantly over the last few years (LI et al., 2021; NASCIMENTO et al., 2021). However, the applications of hydrogels are related to soil conditioning (controlled release of water or nutrients).

Hydrogels can be obtained by chemically or physically crosslinking. The physically crosslinked hydrogels (beads) are more attractive because few reactants and mild conditions are necessary for their synthesis. Using a cationic polymer, like chitosan, with a negatively charged crosslinker (e.g., sodium tripolyphosphate), we may obtain biodegradable beads (PEREZ et al., 2018). However, despite its promising properties, chitosan can make the final product more expensive, limiting its commercial use. An alternative to generate more economically viable beads is replacing chitosan (partial or totally) by another cationic polysaccharide, such as cationic starch. Starch is an abundant biodegradable and cheap polymer found in nature which can be positively charged by alkylation (ANTHONY; SIMS, 2013).

As aforementioned, the maintenance of *A. brasilense* viability and ability to promote plant growth is a challenge. Therefore, in this work, we tested the hypothesis that the beads based on cationic starch can potentiate the effects of *A. brasilense* once it protects the PGPB from adverse soil conditions. To take advantage of the bead's properties and improve the efficiency of *A. brasilense* AbV5/V6, in this work, we described the preparation, characterization, and application of a dual crosslinked hydrogel beads based on cationic starch for encapsulating the PGPB. We investigated the effects of the encapsulation process on the viability of *A. brasilense*. In addition, the effects of *A. brasilense*-loaded beads on maize growth promoting were evaluated by analyzing key biometric parameters and biochemical content in plant growth chamber and greenhouse experiments. Maize was the cereal chosen to validate the technology developed in this work, because it is the dominant grain grown in the world. Maize plays a major role in the livestock industry, biofuel production, and human nutrition. However, less than one-half of applied N as fertilizer is recovered by maize. This unused N by maize poses serious threats to environmental sustainability.

2.2 MATERIALS AND METHODS

2.2.1. Materials

Starch from potato (soluble), (±)-epichlorohydrin (≥99 %, GC), chitosan, CTS (low molecular weight), glutaraldehyde solution (grade II, 25 % in H2O) and sodium tripolyphosphate, STPP (85 %) were purchased from Sigma-Aldrich. Chloridric acid, HCl (37 %) and sodium hydroxide (97 %) were acquired from Fmaia. Absolute ethanol PA EMSURE® ACS, ISO, Reag. Ph. Eur. (>99,9 %) was obtained from Merck. Ethylenediamine (98 %) was purchased from Nuclear, and l-(+)-lactic acid (85 %), from Neon. All reagents were of analytical grade and were used without previous purification.

2.2.2 Preparation of cationic starch

Cationic starch (Starch-NH_2) was obtained following the general procedure reported by (ANTHONY; SIMS, 2013). Firstly, the starch was halogenated by epichlorohydrin through an acid-catalyzed epoxide ring-opening mechanism. Briefly, 10 g of starch was dissolved in 18 mL of epichlorohydrin containing 500 μL of concentrated HCl. The mixture was left reacting under reflux at 110 °C and mild stirring for 24 h. The modified starch was precipitated and washed with ethanol and then dried in a desiccator under a vacuum.

The halogenated starch was then alkylated with ethylenediamine as follows: Approximately 9 g of the halogenated starch was solubilized in 50 mL of 0.16 mol L^{-1} NaOH. Then, 22.5 g of ethylenediamine was added to the solution. The mixture was left reacting under reflux at 60 °C and mild stirring for 24 h. The Starch-NH_2 was precipitated and washed with ethanol, dried in a desiccator under vacuum, and stored until further use.

2.2.3. Synthesis of dual-crosslinked beads

The procedure for the synthesis of dual-crosslinked beads was adapted from (PEREZ et al., 2018) and (WU et al., 2020). Solution "A" was prepared by dissolving 0.2816 g of CTS in 20 mL of lactic acid 1 % (w/v), and Solution "B" by heating to ∼75 °C the starch solution (6.336 g of Starch and 0.4224 g of Starch-NH_2 in 79 mL of distilled water) with constant magnetic stirring. Then, both solutions A and B were mixed and kept under stirring at room temperature for one hour. Finally, 1.126 mL of glutaraldehyde solution 25 % (w/v) was added to the mixture and left to react for an additional 4 h. The blend (Starch/Starch-NH2/chitosan) was used to prepare the beads by a dripping technique. The blend was dripped through plastic tips 2–3 mm in diameter into STPP solution 1 % (w/v). Crosslinking was achieved by leaving the beads overnight in this solution, without stirring. Finally, the beads were removed from the STPP solution, washed with distilled water, and dried at 35 °C for 48 h.

2.2.4. Maize genotype and bacteria strain

Maize (*Zea mays* L.) convencional cultivar BRS Caimbé (Embrapa) and hybrid P3016 (Pioneer) were used in the plant growth chamber and greenhouse experiments, respectively. The tested bacteria were *A. brasilense* strains AbV5 and AbV6 from the commercial inoculant Bioma Mais from Bioma. These strains are authorized for commercial use and are recommended as inoculants for maize, wheat and rice in Brazil (HUNGRIA et al., 2010).

2.2.5. Cells immobilization kinetics and study of the effects of the drying process and storage

A. brasilense AbV5/6 strains were immobilized by immersing sets of five non-sterilized beads (∼19.5 mg, in total) into 3 mL of the commercial inoculant (7.66×10^7 CFU mL^{-1}). Cells immobilization kinetics were evaluated after beads immersion and incubation for 1, 2, 3, 5, 7, 10 and 24 h under agitation (60 rpm) at 30 °C. The amount of *A. brasilense* AbV5/6 immobilized in the beads was estimated either immediately after the mentioned times or after an additional time for drying. Drying process was conducted at 35 °C for 48 h. To determine the number of Colony Forming Unit (CFU) in all tested conditions, a sample of five beads was incubated with 1 mL of sterile saline solution (0.85 % NaCl) for 2 h at room temperature. After that, the beads were grinded and stirred in a vortex for 15 s. Then, the resultant solution was used in a 10-fold serial dilution until 10^{-5} and dropped in RC medium plate (CÁCERES, 1982).

Two biological replicates with three technical replicates were used to define the average number of CFU in each tested condition. Dried beads were evaluated along sixty days of storage, following the procedure just described. The bacteria-loaded beads were stored at room temperature in the absence of light.

2.2.6 Characterizations

2.2.6.1 Fourier transform infrared spectroscopy (FTIR)

All spectra with a spectral range of 4000 to 400 cm^{-1} were recorded in a transmission mode on a Shimadzu FTIR spectrometer (IRPrestige-21). The samples starch and amino starch (starch-NH_2) were analyzed as a dry powder prepared into pellets with KBr in a proportion of 1 mg of sample per 100 mg of KBr. A total of 128 scans were run for each spectrum to set a spectral resolution of 4 cm^{-1}.

2.2.6.2 ^{1}H NMR measurement

^{1}H NMR spectra were recorded on a Bruker Ascend 400 MHz spectrometer by applying a frequency of 400.13 MHz. The acquisition of spectra was carried out using solutions of 20 mg of powdered samples dissolved in 0.6 mL of DMSO-d6 with TMS (0.03 vol%). The analysis conditions involved 16 scans, temperature of 25 °C, spectral window of around 15 ppm, and a d1 of 1 s.

2.2.6.3 Field-emission gun scanning electron microscopy (FEG-SEM)

After the crosslinking reaction, the beads were removed from STPP solution, washed with distilled water, and immediately frozen by immersion in liquid nitrogen, before being lyophilized for 24 h. Under these conditions, it is supposed that the internal structure of the beads remains unchanged. Then, the lyophilized materials were fractured and placed into stubs to expose their internal structure, and afterwards, their surfaces were coated with a thin layer of gold. The SEM images were obtained on a Tescan Mira 3 Scanning Electron Microscope with FEG electron emission source, by applying an acceleration voltage of 15 kV and current intensity of 95 mA.

2.2.6.4 Experimental design

The assay was carried out in a 2 × 2 factorial scheme (with and without hydrogel beads x uninoculated and inoculated with *A. brasilense* AbV5/6), in a completely randomized design, with 4 treatments. The experiment was carried out in a plant growth chamber and replicated in a greenhouse, using soil from a commercial farm.

2.2.6.4.1 Plant growth chamber experiment

Glass test tubes measuring 20 × 250 mm were filled with 25 g of a 1:1 ratio mixture of sieved soil and sand, closed with cotton plug and autoclaved at 121 °C for 40 min. The physical and chemical characteristics of the soil are available in Table S2 (Supplementary data). To ensure the health of the BRS Caimbé maize seeds, they underwent a superficial disinfection process, which consisted of immersion in 95 % ethyl alcohol for 60 s, to facilitate the contact of the seed coat with the hypochlorite solution. Then, a 3 to 6 % sodium hypochlorite solution was placed for 5 min under manual agitation. Finally, the seeds were washed at least six times with autoclaved distilled water (BRAZIL. MINISTRY OF AGRICULTURE, 2010). This entire process was carried out in laminar flow and after the end of the washes, the seeds were placed in sterile Petri dishes for drying at room temperature.

On the day of sowing, the tubes were irrigated to reach 70 % of the field capacity. After percolation of water, one seed per tube was positioned in the center of the tube at 3 cm depth. In treatments containing beads, these were placed to the left of the tube at a depth of 1 cm. For treatments that received the inoculant on the seeds, the concentration was standardized to 1×10^6 CFU per seed. The seeds were packed in plastic bags and the inoculants were added to the seeds with the aid of micropipettes. Then, the plastic bags were manually shaken until the seeds were completely covered. Sowing took place approximately one hour after inoculation.

The soil region of the tubes was covered with kraft paper to prevent contact with light. All tubes were incubated in a plant growth chamber, at a temperature of 25 °C, photoperiod of 12 h and light intensity of 35 to 40 µmoles m^{-2} s^{-1}, where they remained for 10 days.

Ten days after plant emergence, each plant was removed from the tube and separated into shoots and roots. Subsequently, it was performed fresh weight analysis of both parts of the plants. Then, the plants were frozen until root evaluations using an Epson XL 10000 scanner, equipped with an additional light unit coupled to the WinRHIZO Pro 2007a software (Regents Instruments Inc.). Scanner analysis allowed the following morphological characteristics: root length (RL), root surface projection (RSJ), root surface area (RSA), mean root diameter (MRD) and root volume (RV). After carrying out these analyses, the plants were placed to dry in an oven with forced air circulation, until reaching constant mass, and then the dry weight of the aerial part and roots were determined.

2.2.6.4.2 Greenhouse experiment

In the greenhouse, treatments were implanted in 5 L pots filled with soil manually irrigated to maintain the field capacity at 70 %, adding 100 ± 10 mL of water in each pot every 72 h. The chemical characteristics of the soil are available in Table S3 (Supplementary data). Each pot received a hybrid P3016 maize seed chemically treated with the insecticides K'obiol (0.08 mL kg^{-1}), Actellic (0.02 mL kg^{-1}), Dermacor (48 mL per 60,000 seeds) and Poncho (70 mL per 60,000 seeds) and with the fungicides Maxim XL (1.5 mL kg^{-1} of seed) and Derosal Plus (1.5 mL kg^{-1} of seed). The seeds were sown at a depth of 3 cm and the beads were applied at a depth of 1 cm, close to the seeds. The treatments that received the inoculant on the seeds were conducted as described before. Twenty-eight days after sowing, the plants were removed from the pots and the same evaluations mentioned above were performed. In addition to these, when the plant was in the V2 phenological stage, the height of the plants and the levels of chlorophyll a and b were also evaluated. The chlorophyll content was determined using the chlorofiLOG equipment, model CFL 1030 (Falker).

2.2.6.5 Statistical analyses

Variance homoscedasticity was evaluated by Bartlett and model residual normality by Shapiro-Wilk tests. After, an analysis of variance (ANOVA) was performed considering the double factorial design (2 × 2). When significance was verified at 95 % ($p < 0.05$) the differences were tested by Tukey test at 5 % ($p < 0.05$) of significance. All the analyses were performed using R language v. 3.5.1 and ExpDes package (R CORE TEAM, 2018).

2.3 RESULTS AND DISCUSSION

2.3.1 Spectroscopic analysis of amino starch

Before obtaining the cationic starch-based hydrogel beads, the starch was chemically modified with ethylenediamine. Such a modification introduces -NH_2 groups in starch structure which may be easily protonated giving to the polysaccharide a cationic characteristic. Fig. 2.1 shows the FTIR spectra of both starch and NH_2-functionalized starch.

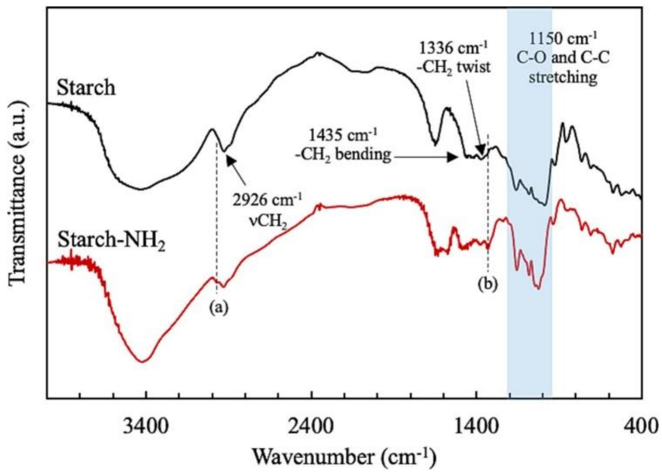

Figure 2.1: FTIR spectra of starch and amino starch (starch-NH_2) in the spectral range of 4000 to 400 cm^{-1}.

The spectra of starch show a broad band in the region between 3200 and 3600 cm^{-1}. This signal was associated with hydrogen bonding water molecules. Another peak related to bending vibration of water molecules can be found at 1644 cm^{-1} (WANG et al., 2022). The signals at 2926 cm^{-1}, 1435, and 1336 cm^{-1} were attributed to the stretching, bending, and twist vibrations of CH_2, respectively (KUMAR et al., 2018). The skeletal mode vibration of the pyranose rings of glucose units were found at 924, and 567 cm^{-1} (SANKARGANESH et al., 2022; WANG et al., 2022). Finally, the bands in the region between 1000 and 1200 cm^{-1} (shaded in light blue) were associated with C-O and C-C stretching.

Comparing the starch-NH_2 to raw starch, the peak at 3400 cm^{-1} became more pronounced. This result was associated to the contribution of νsN-H of primary and secondary amines which is found around 3410 and 3350 cm^{-1}, respectively (AWG SUHAI; CHIN, 2021; SILVERSTEIN, WEBSTER, 2005). Moreover, it can be noted a signal in 2966 cm^{-1} (dashed line a) arising from the asymmetrical and symmetrical stretching in the NH_3^+ group (SILVERSTEIN; WEBSTER, 2005). The signal at 1328 cm^{-1} (dashed line b) was ascribed to methylene twisting vibration characteristic of long chains (TSUBOI et al., 2000; VARMA; VASUDEVAN, 2022). Finally, the signal at around 1560 cm^{-1} was associated with δN-H (CHENG et al., 2009).

To obtain a more comprehensive view of the modification of starch, the samples were also characterized by ^1H NMR (Fig. 2.2). In the spectrum of the starch, the characteristic hydrogen signals were observed. The signals near 5.5 ppm were attributed to hydroxyl groups from C_2 and C_3, while the hydrogen from hydroxyl group of C_6 was found at 4.6 ppm. The peak at 5.1 ppm was associated with the anomeric hydrogen of α-linked glucose residue (H_1). The hydrogens from the pyranosyl ring (H_2 to H_5), and H_6 could be found in the region between 3.0 and 4.0 ppm (ANTUNES et al., 2022; LIMA-TENORIO et al., 2015).

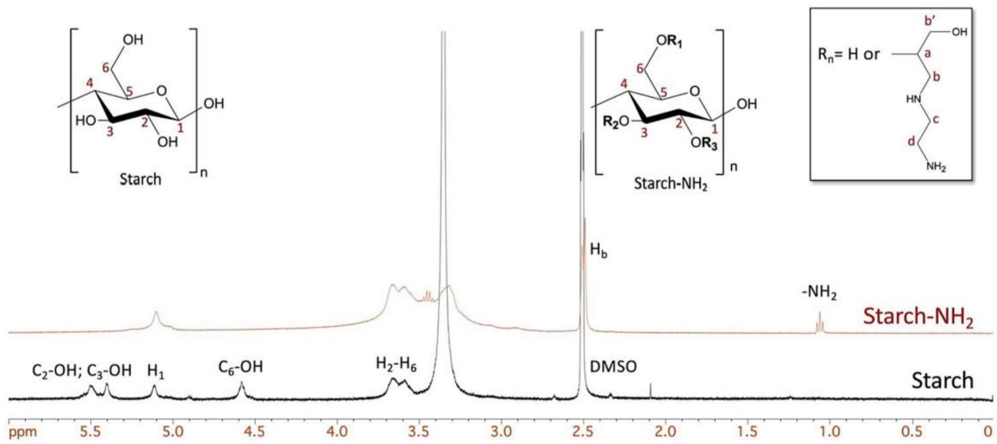

Figure 2.2: ^1H NMR spectra of starch and amino starch (starch-NH_2).

The ^1H NMR spectrum of the modified starch exhibited a new signal at 1.0 ppm which was attributed to hydrogen from primary amine ($-NH_2$) (NOURI et al., 2020). Moreover, the signals relative to hydroxyl groups disappeared, indicating that the chemical modification may occur either in C_2, C_3 or C_6. It is important to point out that the presence of a signal related to H_1 (5.1 ppm) indicates this chemical environment remains the same. As a consequence, we can assume the glycosidic bond was not broken during the chemical modification. In the light of this assessment Fig. 3 shows a simplified schema of the chemical modification route.

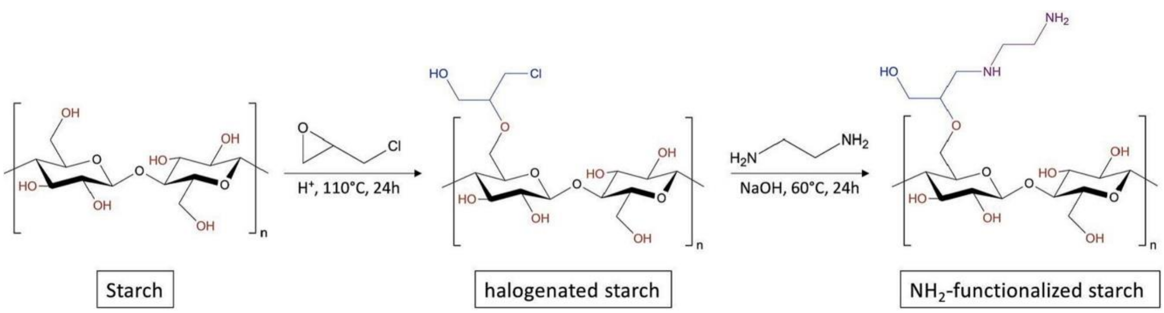

Figure 2.3: Schematic representation of the chemical modification of starch with ethylenediamine. In red: hydroxyl groups which may be modified.

2.3.2 Beads characterization

When immersed in the STPP solution, the mixture of polysaccharides became white with a rounded shape (Fig. 2.4A). However, at this stage, the hydrogel beads showed poor mechanical resistance being easily crumbled. After the crosslinking step, the beads were dried at 40 °C for 24 h. During the drying stage, it is supposed that the crosslinking density increases, due to the approximation of polymeric chains enabling intermolecular interactions. Herein, the starch acts as a filler and may enhance the physical crosslinking by chain entanglement.

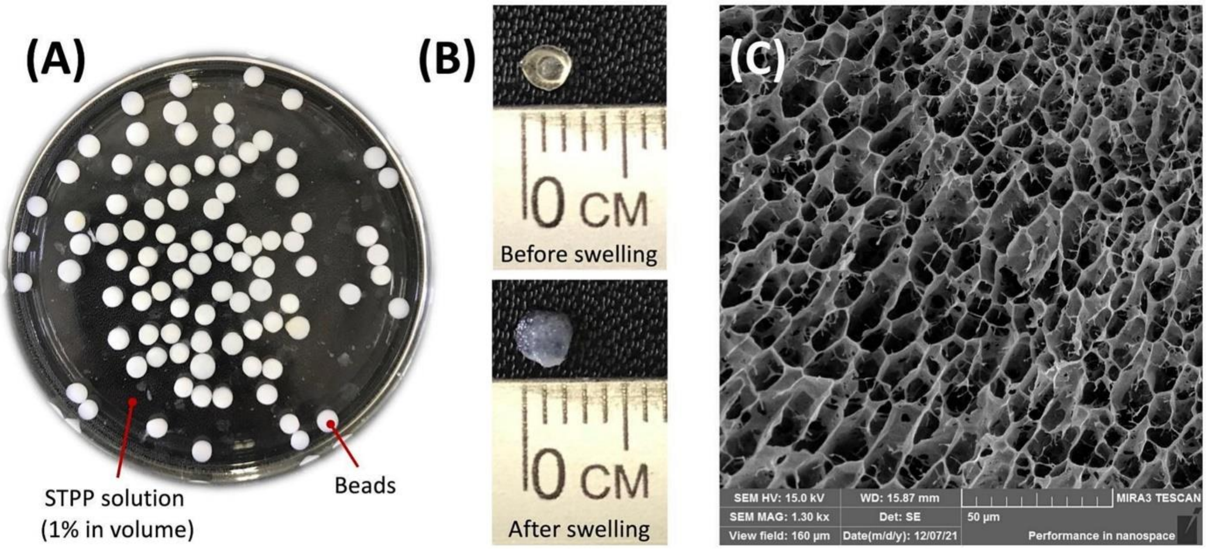

Figure 2.4: The size and shape of the obtained beads: during crosslinking process (A), before and after swelling (B), and their internal structure.

The dried beads showed disk-like shape, and when immersed in distilled water the swollen beads showed improved mechanical resistance although they did not significantly increase their size (Fig. 2.4B).

In any part of the fractured beads, they showed a uniform porous structure typical of hydrogels (Fig. 2.4C). The internal porous structure makes this material attractive to encapsulate and/or release bacteria because they allow diffusion of water into, through, and from the 3D polymer network (LIMA et al., 2018).

2.3.3 Encapsulation and survivor ratio of *A. brasilense*

As mentioned in Section 2.2.5, the *A. brasilense* was encapsulated by a diffusion swelling method. Herein, we investigated the effect of contact time of the beads with the culture medium (7.66×10^7 CFU mL^{-1}), as well as, the effect of drying the bacteria-loaded beads on the viability of the AbV5/6. The total number of viable bacteria in the beads was measured in specific time intervals. The results are shown in Fig. 2.5.

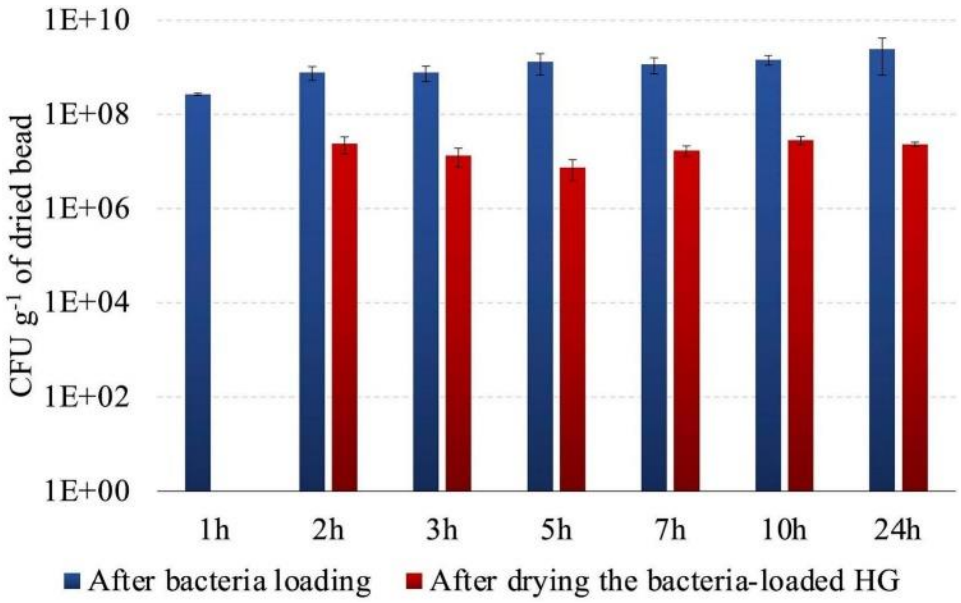

Figure 2.5: Quantification of *A. brasilense* (AbV5/6) encapsulated at different times in the hydrogel beads: Immediately after encapsulation procedure (blue), and after encapsulating and drying the beads at 35 °C for 48 h (red). Average values obtained from two independent biological replicates each of them comprising three technical replicates.

As a general trend, the higher the contact time of beads with the culture medium, the better the encapsulation efficiency. In fact, the time dependence is close to linear behavior. After 24 h, it achieved a maximum of encapsulated bacteria near 2.44×10^9 CFU g^{-1} of dried beads (Fig. 2.5, blue bars).

The drying process is a critical step that decreases the bacteria viability but on the other hand, it increases biological products shelf life and decreases contamination with undesired microorganisms (DUARTE et al., 2022; PEREZ et al., 2018). To evaluate this effect, for each specific time interval some bacteria-loaded beads were dried at 35 °C for 48 h. When the encapsulation time was 1 h, none bacteria survived the drying process, indicating that most bacteria were immobilized only on the bead surface. As a consequence, the *A. brasilense* was not entirely protected. On the other hand, in the subsequent encapsulation times, the drying process decreased the bacteria viability by two orders of magnitude (10^2 UFC g^{-1}).

The effect of the storage was also evaluated along sixty days (Fig. 2.6). After the mentioned time of storage, the viable bacteria were quantified by measuring the CFU g^{-1} of dried beads.

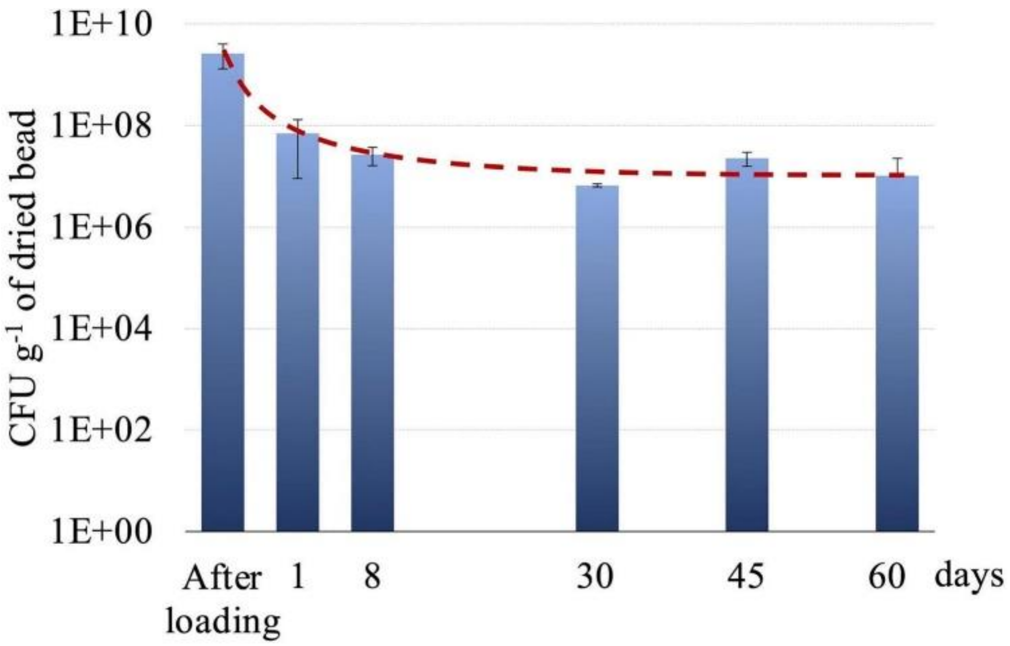

Figure 2.6: Viability of encapsulated *A. brasilense* (AbV5/6) into dried beads stored at room temperature for 60 days. The average values were obtained from two independent biological replicates each of them comprising three technical replicates.

The total number of viable bacteria measured immediately after loading was found to be in the order of 10^9 CFU g^{-1}. After drying and one day of storage the population of viable bacteria decreased to 10^7 CFU g^{-1}. As aforementioned, a reduction in two orders of magnitude is associated with the kill of most of bacteria present in the bead surface that do not survive the drying approach and storage. In the subsequent days, the average of viable bacteria remains in the same order of magnitude (10^7 CFU g^{-1}), indicating that the hydrogel beads are suitable for protecting the *A. brasilense* for a period of at least 60 days.

Based on these results, for the subsequent investigations all bacteria-loaded beads were prepared by immersing the beads in a suspension solution of *A. brasilense* (7.66×10^7 CFU mL^{-1}) during 24 h, followed by a drying step at 35 °C for 48 h. In addition, those encapsulated beads were tested within 60 days of storage.

2.3.4 Effect of encapsulated bacteria inoculation under plant growth chamber experiment

Under Plant Growth Chamber conditions, the presence of bead induced a 17 % increase of shoot fresh weight (SFW) when compared to the control without the bead (Fig. 2.7A). Analyzing only the inoculant factor, non-inoculated control plants presented SFW statistically superior to those inoculated with *A. brasilense* AbV5/6 (Fig. 2.7B). For RL, the interaction between inoculation and bead was significant. The inoculation of AbV5/6 encapsulated into hydrogel bead increased RL (Fig. 2.7C). Plants inoculated with encapsulated *A. brasilense* into beads presented root length, on average, 19 % higher than the non-inoculated ones, showing that this technology can be useful in improving the performance of inoculated plants.

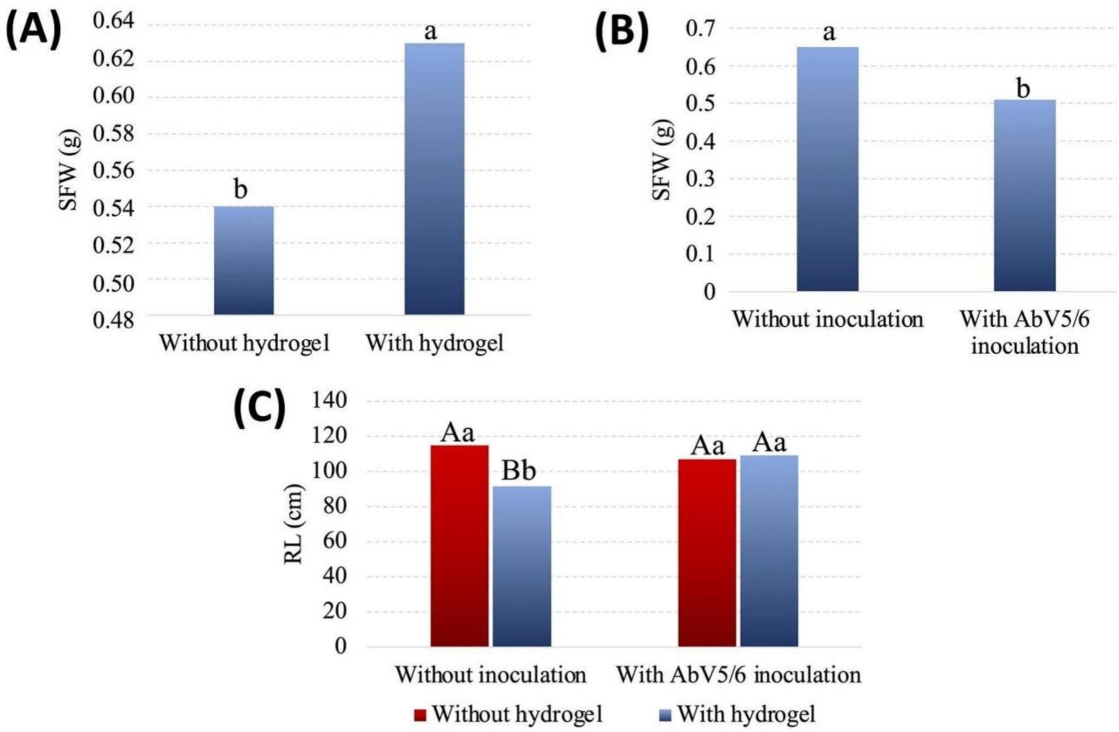

Figure 2.7: Effect of bead use on shoot fresh weight (SFW) (A), effect of inoculation factor on shoot fresh weight (SFW) (B), and combined effect of inoculation and bead on root length (RL) (C) parameters at 10 days after emergence of maize grown under plant growth chamber experiment. In A and B, equal letters within each graph are not statistically different (Tukey, $p \leq 0.05$). In (C), capital letters represent the statistic within the inoculation factor and lowercase letters represent the statistic within the bead factor. When followed by the same letter, within each factor are not statistically different (Tukey, $p \leq 0.05$).

The results found by (MEFTAH KADMIRI et al., 2021), confirmed the existence of an interaction between bacteria and the encapsulation matrix composition, which affects the release behavior, stability of survival and consequently the effect of the biofertilizer on the plants. In this work, the experimental tests showed results both in the aerial part of maize plants, as well as in the roots, suggesting a promising effect of the use of the bead associated with inoculation with *A. brasilense*.

2.3.5 Effect of encapsulated bacteria inoculation under greenhouse experiment

For the greenhouse experiment, only the variable chlorophyll showed statistically significant interaction between inoculation and hydrogel bead. The inoculation increased the chlorophyll a and b content; however, this effect was only observed when the beads were present (Fig. 2.8). It is worth mentioning that the seeds used in the experiment in a greenhouse were chemically treated, which may have affected the viability of *A. brasilense* AbV5/6 bacteria, when in the absence of the beads. According to (SZOPA et al., 2022), the beads were designed to create a temporary barrier to microorganisms and provide a microenvironment that supports its viability, where its matrix is degraded due to soil bacterial activity, ensuring a slow release of the inoculant into the soil, thus increasing its efficiency for a longer period.

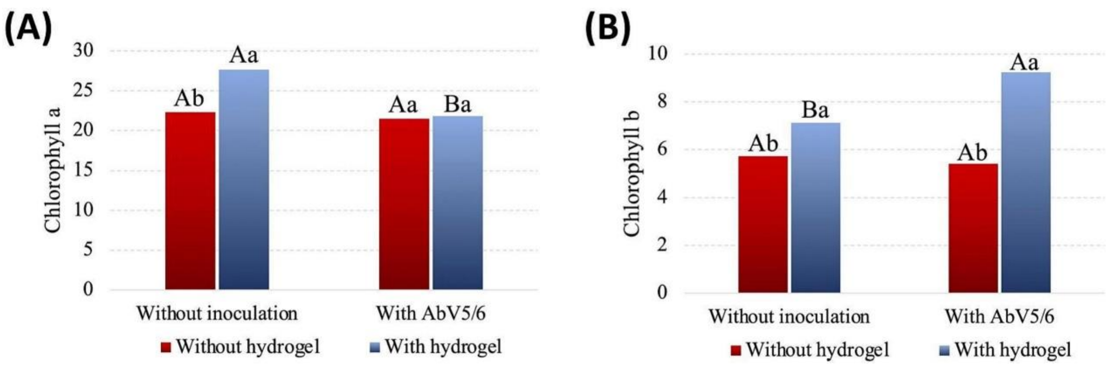

Figure 2.8: Effect of the interaction between inoculation and beads on the chlorophyll a (A) and b (B) content of maize leaves evaluated at the V2 phenological stage, in the greenhouse experiment. Values obtained according to Falker chlorophyll index. Capital letters represent the statistic within the inoculation factor and lowercase letters represent the statistic within the beads factor. When followed by the same letter, within each factor are not statistically different (Tukey, $p \leq 0.05$).

Beads provided an increase in the chlorophyll a only when the plants were not inoculated (Fig. 2.8A). However, the presence of beads increased chlorophyll b content of both non-inoculated and inoculated treatments. The encapsulated formulation of *A. brasilense* AbV5/6 increased approximately 30 % of chlorophyll b content compared to the non-inoculated bead. In addition, the encapsulation of *A. brasilense* AbV5/6 into the beads increased the chlorophyll b content in 71 % (Fig. 2.8B). Since nitrogen is one of the main components of chlorophyll structure, the nitrogen fixation provided by *A. brasilense* AbV5/6 could be increasing the amount of nitrogen uptake by the plant, and therefore the chlorophyll content, the photosynthesis rate and biomass of the plant (MIRSHEKARI et al., 2015).

The parameters which showed a single effect of inoculation or beads were SFW and plant height. The presence of hydrogel beads resulted in benefits of 6 % and 7 %, in the plant

SFW and height, respectively, as shown in Fig. 2.9. Due to the hydrophilic nature of hydrogels, it may act as mini water reservoirs, reducing compaction, and increasing soil aeration, inducing plants to higher growth rate.

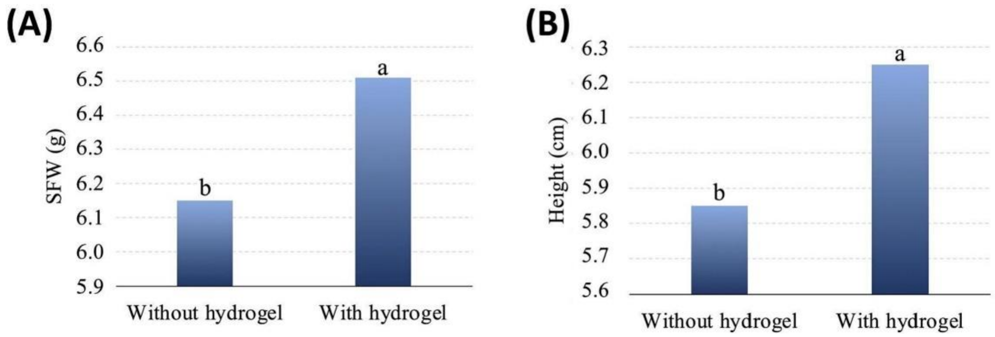

Figure 2.9: Effect of beads on shoot fresh weight (SFW) (A) and maize height at V2 phenological stage (B), in a greenhouse experiment. Equal letters within each graph are not statistically different (Tukey, $p \leq 0.05$).

2.4 CONCLUSIONS

Cationic starch was synthesized by the halogenation of starch, followed by the alkylation of amines. Then, dual-crosslinked hydrogel beads were prepared by an easy dripping technique. The *A. brasilense* was successfully encapsulated into the beads, and cells immobilization kinetics showed that, after 24 h, a maximum of encapsulated bacteria was achieved (near 2.44×10^9 CFU g^{-1} of dried beads), although the drying process decreased the bacteria viability by two orders of magnitude (10^2 UFC g^{-1}). The beads are shown to be non-toxic, while keeping the encapsulated bacteria viable for a long period of time (at least 60 days).

The *A. brasilense* AbV5/6-loaded beads were tested to promote maize growth in a plant growth chamber and greenhouse and showed promising results for plant development. Under controlled conditions, the use of beads provided an increase in shoot fresh mass and increased root length by 19 %, which benefits the plant in the acquisition of nutrients and water, obtaining better performances. In a greenhouse, the association of inoculation with AbV5/6 with the hydrogel beads improved the chlorophyll levels of maize plants, the most significant effect being for the levels of chlorophyll b. The use of beads contributed to the increase in shoot fresh mass and height of maize plants.

The presented biometric and biochemical results showed the potential of the technology developed in this work to enhance the effects of *A. brasilense* AbV5/6 in promoting maize growth in controlled conditions using polysaccharides from renewable sources and abundant in nature.

2.5 ACKNOWLEDGEMENTS

Brazilian Program of National Institutes of Science and Technology-INCT/Brazilian Research Council-CNPq/MCT, National Council for Scientific and Technological Development – CNPq (grant numbers 406008/2022-1; 304524/2019-0; 306641/2022-4; 404404/2021-9), CAPES and Fundação Araucária (grant number JDT2022271000043) of the Paraná State for the financial support. The authors would like to thank the multi-user laboratory (C-LABMU), LMPM and LABMOM team from State University of Ponta Grossa (UEPG) – Brazil for the equipment and analysis, and also the Simbiose company.

REFERENCES

ANTHONY, R. J.; SIMS, R. C. Optimization of cationic amino starch synthesis using biogenic amines. **Carbohydrate Polymers**, v. 98 (2), p. 1409–1415, 2013.

ANTUNES, B. F. et al. Crosslinking starch with Diels-Alder reaction: Water-soluble materials and water-mediated processes. **Polymer International**, v. 71, p. 1340-1346, 2022.

AWG SUHAI, A. A. K.; CHIN, S. Green synthesis and characterization of amine- modified starch nanoparticles. **Starch-Starke**, v. 73, n. 1–2, Article 2000020, 2021.

BASHAN, Y.; DE-BASHAN, L. E. In How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—A critical assessment. **Advances in Agronomy**, v. 108, p. 77–136, 2010.

CÁCERES, E. A. R. Improved medium for isolation of *Azospirillum* spp. **Applied and Environmental Microbiology**, v. 44, n. 4, p. 990–991, 1982.

CANFIELD, D. E.; GLAZER, A. N.; FALKOWSKI, P. G. The evolution and future of Earth's nitrogen cycle. **Science**, v. 330, p. 192–196, 2010.

CHENG, R. et al. Ethylenediamine modified starch as biosorbent for acid dyes. **Journal of Hazardous Materials**, v. 172, n. 2–3, p. 1665–1670, 2009.

Brazil. Ministry of Agriculture, Cattle and Supplying. (2010). **Normative Instruction n° 30**. December 12th, 2010. Available at http://www.agricultura.gov.br/assuntos/insumos-agropecuarios/insumos-agricolas/fertilizantes/legislacao/in-30-2010-dou-17-11-10-metodo inoculantes.pdf. Access on 15/12/2023.

D'AYALA, G. G.; MALINCONICO, M.; LAURIENZO, P. Marine derived polysaccharides for biomedical applications: Chemical modification approaches. **Molecules**, v. 13, n. 9, p. 2069–2106, 2008.

DERAKHSHANKHAH, H. et al. Folate-conjugated thermal- and pH-responsive magnetic hydrogel as a drug delivery nano-system for "smart" chemo/hyperthermia therapy of solid tumors. **Materials Today Communications**, v. 30, Article 103148, 2022.

DÍAZ-ZORITA, M. et al. (2015). Field Evaluation of Extensive Crops Inoculated with *Azospirillum* sp.. In: CASSÁN, F.; OKON, Y.; CREUS, C. (eds) **Handbook for Azospirillum**. Springer, Cham.

DUARTE, M. M. et al. Contributions of supercritical fluid technology for advancing decellularization and postprocessing of viable biological materials. **Materials Horizons**, v. 9, n. 3, p. 864–891, 2022.

FOLEY, J. A. et al. Solutions for a cultivated planet. **Nature**, v. 478, n. 7369, p. 337–342, 2011.

GROVER, M. et al. PGPR mediated alterations in root traits: Way toward sustainable crop production. **Frontiers in Sustainable Food Systems**, v. 4, 2021.

HUNGRIA, M. et al. Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. **Plant and Soil**, v. 331, p. 413–425, 2010.

KHAN, F. et al. Synthesis, classification and properties of hydrogels: Their applications in drug delivery and agriculture. **Journal of Materials Chemistry B**, v. 10, n. 2, p. 170–203, 2022.

KUMAR, S. V.; KUMAR, V. A. S.; KUMAR, S. The influence of bound water on the FTIR characteristics of starch and starch nanocrystals obtained from selected natural sources. **Starch-Starke**, Article 1700026, 2018.

LI, Y. et al. Study on preparation and application of a multifunctional microspheric soil conditioner based on arabic gum, gelatin, chitosan and β-cyclodextrin. **International Journal of Biological Macromolecules**, v. 183, p. 1851–1860, 2021.

LIMA, D. S. et al. pH-responsive alginate-based hydrogels for protein delivery. **Journal of Molecular Liquids**, v. 262, p. 29–36, 2018.

LIMA-TENORIO, M. K. et al. Water transport properties through starch-based hydrogel nanocomposites responding to both pH and a remote magnetic field. **Chemical Engineering Journal**, v. 259, p. 620–629, 2015.

MAITZ, M. F. Applications of synthetic polymers in clinical medicine. **Biosurface and Biotribology**, v. 1, n. 3, p. 161–176, 2015.

MEFTAH KADMIRI, I. et al. Bioformulation of microbial fertilizer based on clay and alginate encapsulation. **Current Microbiology**, v. 78, n. 1, p. 86–94, 2021.

MIRSHEKARI, B. et al. *Azospirillum lipoferum* and nitrogen fertilization effect on chlorophyll content, nutrients uptake and biometric properties of *Zea mays* L. **Agrociencia**, v. 49, n. 8, p. 889–897, 2015.

NASCIMENTO, C. D. V. et al. Potential of superabsorbent hydrogels to improve agriculture under abiotic stresses. **Journal of Arid Environments**, v. 189, Article 104496, 2021.

NOURI, A. et al. Diaminated starch: A competitor of chitosan with highly mucoadhesive properties due to increased local cationic charge density. **Biomacromolecules**, v. 21, n. 2, p. 999–1008, 2020.

PASTOR, A.V. et al. The global nexus of food–trade–water sustaining environmental flows by 2050. **Nature Sustainability**, v. 2, n. 6, p. 499–507, 2019.

PEDROSA, F. O. et al. The ammonium excreting *Azospirillum brasilense* strain HM053: A new alternative inoculant for maize. **Plant and Soil**, v. 451, n. 1–2, p. 45–56, 2020.

PELLÁ, M. C. G. et al. Magnetic chitosan microgels: Synthesis, characterization, and evaluation of magnetic field effect over the drug release behavior. **Carbohydrate Polymers**, v. 250, Article 116879, 2020.

PEREG, L.; DE-BASHAN, L. E.; BASHAN, Y. Assessment of affinity and specificity of *Azospirillum* for plants. **Plant and Soil**, v. 399, n. 1–2, p. 389–414, 2016.

PEREZ, J. J. et al. A novel, green, low-cost chitosan-starch hydrogel as potential delivery system for plant growth-promoting bacteria. **Carbohydrate Polymers**, v. 202, p. 409–417, 2018.

PONGSILP, N.; NIMNOI, P. Inoculation of Ensifer fredii strain LP2/20 immobilized in agar results in growth promotion and alteration of bacterial community structure of Chinese kale planted soil. **Scientific Reports**, v. 10, n. 1, Article 15857, 2020.

QAMAR, S. A. et al. Alginate-based nano-adsorbent materials – Bioinspired solution to mitigate hazardous environmental pollutants. **Chemosphere**, v. 288, Article 132618, 2022.

R CORE TEAM. **R: A language and environment for statistical computing.** Vienna, Austria: R Foundation for Statistical Computing. URL https://www.R-project.org/, 2018.

RICHARDSON, Y.; BLIN, J.; JULBE, A. A short overview on purification and conditioning of syngas produced by biomass gasification: Catalytic strategies, process intensification and new concepts. **Progress in Energy and Combustion Science**, v. 38, n. 6, p. 765–781, 2012.

SANKARGANESH, P. et al. Preparation of PVA/starch hydrogel and its in-vitro drug release potential against pus-inducing pathogenic strain and breast cancer cell line. **Journal of Sol-Gel Science and Technology**, v. 101, n. 3, p. 571–578, 2022.

SHAH, A. et al. PGPR in agriculture: A sustainable approach to increasing climate change resilience. **Frontiers in Sustainable Food Systems**, v. 5, 2021.

SILVERSTEIN, R. M.; WEBSTER, F. X. (2005). **Spectrometric identification of organic compounds**. Wiley, 502 p. https://books.google.com.br/books?id=3VJCAQAAIAAJ.

SMITH, P. et al. How much land-based greenhouse gas mitigation can be achieved without compromising food security and environmental goals? **Global Change Biology**, v. 19, n. 8, p. 2285–2302, 2013.

SUPARE, K.; MAHANWAR, P. Starch-chitosan hydrogels for the controlled-release of herbicide in agricultural applications: A study on the effect of the concentration of raw materials and crosslinkers. **Journal of Polymers and the Environment**, v. 30, n. 6, p. 2448–2461, 2022.

SZOPA, D. et al. Encapsulation efficiency and survival of plant growth- promoting microorganisms in an alginate-based matrix – A systematic review and protocol for a practical approach. **Industrial Crops and Products**, v. 181, Article 114846, 2022.

TAKAHASHI, W. Y. et al. Impact of seed-applied fungicide and insecticide on *Azospirillum brasilense* survival and wheat growth-promoting ability. **Letters in Applied Microbiology**, v. 74, n. 4, p. 604–612, 2022.

TIMMUSK, S. et al. Perspectives and challenges of microbial application for crop improvement. **Frontiers in Plant Science**, v. 8, 2017.

TSUBOI, M. et al. Intensity of the polarized Raman band at 1340–1345 cm - 1 as an indicator of protein α-helix orientation: Application to Pf1 filamentous virus. **Biochemistry**, v. 39, n. 10, p. 2677–2684, 2000.

URREA-VALENCIA, S. et al. Detection of *Azospirillum brasilense* by qPCR throughout a maize field trial. **Applied Soil Ecology**, v. 160, Article 103849, 2021.

VARMA, R.; VASUDEVAN, S. Synthesis and characterization of cellulose and cellulose nanocrystals from dead seagrass – Towards the wealth from waste concept. **Cellulose Chemistry and Technology**, v. 56, n. 1–2, p. 39–47, 2022.

WANG, X. et al. Cationic starch modified bentonite-alginate nanocomposites for highly controlled diffusion release of pesticides. **International Journal of Biological Macromolecules**, v. 213, p.123–133, 2022.

WU, T. et al. Preparation, characterization, and release behavior of doxorubicin hydrochloride from dual cross-linked chitosan/alginate hydrogel beads. **ACS Applied Bio Materials**, v. 3, n. 5, p. 3057–3065, 2020.

GENERAL CONCLUSION

The new strains of *A. brasilense* showed variations in responses, depending on the location of the experiment and the evaluated phenological stage. In general, the new strains were sometimes equal to the performance found by co-inoculation with *A. brasilense* AbV5/6, sometimes they presented results superior to this one. The main highlight was the results found with the new strains at Mandaguaçu, where they presented a statistically superior performance to the controls and co-inoculation with AbV5/6, with the strain *A. brasilense* HM053 always being the best among them, with an increase of more than 90% in the dry weight of nodules and of 21.7% of increase in the productivity in comparison to the standard co-inoculation with AbV5/6. At Dois Vizinhos, strain HM053 and IH1 also stood out for nodulation parameters in the flowering stage, with strain HM053 again showing the highest values of NN and NDW.

At Pindorama, all co-inoculation combinations with strains of *A. brasilense* showed a performance equal to that found by the treatment that received only mineral fertilizer for the TSNC, showing that these bacteria are effective in providing the nitrogen necessary for the development of soybean plants.

At Pindorama, Rio Verde and Indiara all new strains were statistically equal to co-inoculation with AbV5/6. At Dois Vizinhos, the best productivity was found with the single inoculation with *Bradyrhizobium.*

Despite the differences in climate and soil found in the experimental sites, the new strains of *A. brasilense* showed satisfactory and promising results. However, due to these differences in the response pattern, more studies must be conducted in the soybean crop, in order to obtain a more accurate result.

Furthermore, these new strains must be tested in new formulations, with the aim of improving their viability and efficiency in the field. For this, we carried out the experiments carried out in the second chapter of the thesis, where it was possible to encapsulate *A. brasilense* AbV5/6 in hydrogel beads, and its viability was effective for at least 60 days of storage, maintaining its concentration in the order of magnitude 10^7 CFU g^{-1}.

The encapsulated bacteria showed good results for growth parameters of maize plants, obtaining increases of 19% in root length, 17% increase in fresh weight of aerial art and 71% in chlorophyll b content, proving to be an innovative and efficient technology with great perspective of use. For this, new experiments must be conducted, with other crops of agricultural interest and under field conditions, so that it is possible to validate this new application technology and improve the performance of the inoculated strains, favoring their viability and stability despite biotic and abiotic stresses found in soils.

ATTACHMENTS

Attached Table S1: Soil chemical and physical properties, and population of soybean bradyrhizobia and diazotrophic bacteria of the soil in the 0-20 cm layer. Soil collected before sowing the experiment in the plant growth chamber.

Site	**Depth**	**pH**	**(H+Al)**	**Al**	**Ca**	**Mg**	**K**	**CTC**	**P**	**C**	**V**	**Sand**	**Silt**	**Clay**	***Bradyrhizobium***	**Diazotrophic bacteria**
	cm	$CaCl_2$			cmol$_c$ dm^{-3}				mg dm^{-3}	g dm^{-3}	%		g kg^{-1}		Cells/g soil	
Mandaguaçu	0-20	5.30	5.23	0.00	4.08	1.17	0.65	11.13	12.42	21.34	53.01	300	60	640	7.8×10^2	6.25×10^3
Dois Vizinhos	0-10	5.70	3.74	0.00	6.50	3.10	0.23	13.57	12.70	21.00	72.43	58.93	145.55	795.52	0.0	6.5×10^3
Pindorama	0-20	5.59	1.07	NI	1.43	0.45	0.34	3.29	10.29	9.08	67.48	892	36	72	NI	NI
Rio Verde	NI	6.10*	NI	0.03	4.40	1.30	0.65	6.38**	15.20	20.24	55.60	362	174	464	4.8×10^3	NI
Indiara	NI	6.30*	NI	0.00	3.80	1.10	0.00	4.9**	29.60	12.99	65.00	610	150	240	2.6×10^4	NI

*pH in water; **effective CTC. NI – Not Informed.

Attached Table S2: Chemical and physical properties of the soil in the 0-20 cm layer. Soil collected before sowing the experiment in the plant growth chamber. Ponta Grossa, 2022.

CHEMICAL											**PHYSICAL**		
$CaCl_2$	**$cmol_c\ dm^{-3}$**							**%**	**$g\ dm^{-3}$**	**$mg\ dm^{-3}$**	**$g\ kg^{-1}$**		
pH	(H+Al)	K	Ca	Mg	Al	CEC	T_{CEC}	BS	MO	P*	Clay	Silt	Sand
5.01	5.11	0.13	7.27	3.42	0.0	15.93	10.82	67.9	40.18	4.53	64	24	12

Legend: CEC - Cation Exchange Capacity (H+Al+Ca+Mg+K); T_{CEC} - Ca+Mg+K; BS - Base Saturation (T_{cec}/CEC) × 10. *P extracted with Mehlich-1 solution.

Attached Table S3: Chemical properties of the soil in the 0-20 cm layer. Soil collected before sowing the experiment in the greenhouse. Ponta Grossa, 2022.

$CaCl_2$	**$cmol_c\ dm^{-3}$**							**%**	**$g\ dm^{-3}$**	**$mg\ dm^{-3}$**
pH	**(H+Al)**	**K**	**Ca**	**Mg**	**Al**	**CEC**	**T_{CEC}**	**BS**	**MO**	**P***
5.00	4.13	0.31	2.17	1.10	0.03	7.71	3.58	46.4	18.40	11.60

Legend: CEC - Cation Exchange Capacity (H+Al+Ca+Mg+K); T_{CEC} - Ca+Mg+K; BS - Base Saturation (T_{cec}/CEC) × 10. *P extracted with Mehlich-1 solution. The soil was classified as a dark red oxisol, with medium texture, with clay content of 26.5% and smooth wavy relief.