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Azospirillum brasilense **ESTIRPES CNPSo 2083 (=Ab-V5) E CNPSo 2084 (=Ab-V6):** PROMOÇÃO DO CRESCIMENTO DE PLANTAS E AVALIAÇÃO DA COMPATIBILIDADE COM AGROTÓXICOS UTILIZADOS NO TRATAMENTO DE SEMENTES DE MILHO (*Zea mays* L.)

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade Estadual de Londrina - UEL, como requisito à obtenção do título de Doutora.

Orientadora: Drª. Mariangela Hungria

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“Não fui eu quem ordenou a você que seja forte e corajoso? Não tenha medo e não se sinta acovardado, porque Javé seu Deus vai estar com você por onde você andar”. Josué 1:9.

SANTOS, Mariana Sanches. ***Azospirillum brasilense* estirpes CNPSo 2083 (=Ab-V5) e CNPSo 2084 (=Ab-V6):** Promoção do crescimento de plantas e avaliação da compatibilidade com agrotóxicos utilizados no tratamento de sementes de milho (*Zea mays* L.). 2021. 138 f. Tese (Doutorado em Biotecnologia) – Universidade Estadual de Londrina, Londrina, 2021.

RESUMO

A humanindade discute a preservação ambiental, que é um desafio frente ao desenvolvimento há pelo menos um século. Nas últimas décadas o setor agrícola tem buscado criar novas práticas que aliam sustentabilidade ambiental com altos índices de produtividade e de qualidade dos alimentos. Os inoculantes são um exemplo de bioinsumo de aplicação agrícola que reúnem essas características, garantindo produtividade elevada com redução no uso de fertilizantes químicos, altamente poluentes. O Brasil tem se destacado no uso de inoculantes em diversas culturas, como a soja, o feijão e o milho. Por décadas, os inoculantes usados no país eram à base de rizóbios para leguminosas mas, há pouco mais de uma década, entraram e ganharam mercado inoculantes com as estirpes Ab-V5 (=CNPSo 2083) e Ab-V6 (=CNPSo 2084) da bactéria promotora do crescimento de plantas (BPCP) *Azospirillum brasilense*. Contudo, ainda há poucos estudos sobre esses inoculantes. Para garantir um bom desempenho do inoculante é necessário que as células estejam viáveis e em uma concentração elevada. Diante disso, pesquisadores e produtores têm questionado se o tratamento de sementes com agrotóxicos, prática comum e, na maioria dos casos, indispensável para a proteção contra pragas e doenças, poderia interferir nos resultados da inoculação. Os objetivos deste trabalho foram descrever aspectos importantes referentes à tecnologia da inoculação e avaliar os impactos que o tratamento de sementes com agrotóxicos pode causar às células de *A. brasilense* e, consequentemente, na sua contribuição à agricultura. O primeiro trabalho, uma revisão, descreve o cenário mundial de uso de inoculantes nas mais diversas culturas, os microrganismos utilizados e técnicas de inoculação. Na segunda revisão foram discutidos os principais estudos realizados no Brasil, a situação comercial atual e as expectativas sobre o uso das estirpes Ab-V5 e Ab-V6 como inoculantes nas diversas culturas. O terceiro trabalho constou no desenvolvimento de uma nova metodologia para verificar a recuperação de células viáveis de *A. brasilense* em sementes de milho inoculadas, impressindível para a avaliação dos efeitos do tratamento de sementes com agrotóxicos. No quarto trabalho, a nova metodologia foi aplicada e foram descritos os efeitos que o uso de agrotóxicos pode desencadear no desenvolvimento inicial e na morfologia das raízes de milho inoculado ou não com *A. brasilense*. No quinto trabalho foram revisados e discutidos trabalhos relatando níveis de compabitilidade ou incompatibilidade entre inoculantes e agrotóxicos (fungicidas, inseticidas e herbicidas). Os resultados obtidos evidenciaram a relevância crescente que os inoculantes microbianos vêm adquirindo na agricultura nacional e internacional, e o impacto positivo que, em uma década de uso, as estirpes Ab-V5 e Ab-V6 promoveram tanto em leguminosas, como em gramíneas. Contudo, os resultados indicam também que agrotóxicos utilizados no tratamento de sementes representam uma ameaça aos benefícios que podem ser obtidos pela inoculação com *Azospirillum*, afetando negativamente desde a sobrevivência das células, ao desenvolvimento radicular e rendimento das culturas. Em cada um dos cinco estudos são sugeridas pesquisas futuras e estratégias para incrementar a contribuição dos inoculantes na agricultura brasileira.

Palavras-chave: Inoculantes. BPCP. Fixação biológica de nitrogênio. Agrotóxicos.

SANTOS, Mariana Sanches. ***Azospirillum brasilense* strains Ab-V5 and Ab-V6:** Plant growth-promotion and compatibility with pesticides used in the seed treatment of maize (*Zea mays* L.). 2021. 138 pp. Thesis (Doctorate degree in Biotechnology) – Universidade Estadual de Londrina, Londrina, 2021.

ABSTRACT

The environment preservation has been discussed for at least a century. In the last decades, the agricultural sector has sought to create new practices that combine environmental sustainability with high yields and food quality. The inoculants are examples of bio-inputs for agricultural application that combine these characteristics, guaranteeing high yield with less use of chemical fertilizers, which are highly polluting. Brazil has excellency in the use of inoculants in several crops, such as soybeans, common beans and maize. For decades, the inoculants used in the country were based exclusively on rhizobia for legumes, but a decade ago, the strains Ab-V5 (= CNPSo 2083) and Ab-V6 (= CNPSo 2084) of the plant-growth-promoting bacterium (PGPB) *Azospirillum brasilense* were released and started to occupy an important space in the commercial market. However, there are still few studies on these inoculants. In order to guarantee a good performance of the inoculant, the cells must be viable at high concentration. Therefore, researchers and farmes have argued if seed treatment with pesticides, a common and indispensable practice against pests and diseases can interfere in the results of inoculation. The objectives of this study were to describe important aspects related to the inoculation technology and to evaluate the impacts that seed treatment with pesticides can cause on *A. brasilense* cells and, consequently, on its contributions in agriculture. In the first review work, the world scenario of the use of inoculants in a variety of crops, of inoculation techniques and microorganisms used was described. In the second review, the main studies carried out in Brazil, the current commercial situation and expectations about the use of strains Ab-V5 and Ab-V6 as inoculants in different crops were discussed. The third study describes the development of a new methodology to verify the recovery of viable cells of *A. brasilense* in inoculated seeds of maize, essential for evaluating the effects of seed treatment with pesticides. In the fourth study, the new developed methodology was applied to verify the effects of pesticides on the initial development and on the morphology of maize roots inoculated or not with *A. brasilense*. In the fifth work, papers reporting levels of compatibility or incompatibility between inoculants and pesticides (fungicides, insecticides and herbicides) were reviewed and discussed. The results obtained show the growing relevance that microbial inoculants have played in the Brazilian agriculture, and the impact that, in a decade of use, the strains Ab-V5 and Ab-V6 promoted as inoculants of both legumes and non-legumes. However, the results also indicate that pesticides used in seed treatment represent a threat to the benefits that can be obtained by inoculation with *Azospirillum*, affecting not only cell survival but also root development and crop yield. In each study, strategies are suggested to increase the contribution of inoculants to the agriculture in Brazil.

Key-words: Inoculant. PGPB. Biological nitrogen fixation. Pesticides

SUMÁRIO

INTRODUÇÃO

Os inoculantes, bioprodutos agrícolas capazes de substituir total ou parcialmente os fertilizates químicos, têm ganhado cada vez mais notoriedade no agronegócio. Contendo microrganismos vivos, esses produtos favorecem o desenvolvimento de plantas por meio de diversos mecanismos, permitindo elevada produtividade a baixo custo. Desde quando chegou ao mercado pela primeira vez, em 1896, o número de doses comercializadas aumentou consideravelmente e o cenário atual indica continuidade de crescimento, devido à divulgação dos excelentes resultados e do desenvolvimento de novas formulações para diversas culturas.

O gênero *Azospirillum* abrange um grupo de bactérias Gram-negativas, que fazem parte da subclasse α das proteobactérias. Diazotróficas associativas, também podem viver livremente no solo na forma de cistos (MOREIRA et al., 2010). Bactérias do gênero *Azospirillum* estão distribuídas em diversas regiões do mundo, em condições tropicais, subtropicais e temperadas (HUNGRIA, 2011; SIVASAKTHIVELAN; SARANRAJ, 2013), em associação com plantas monocotiledôneas e eudicotiledôneas (STEENHOUDT; VANDERLEYDEN, 2000). O Brasil foi pioneiro em estudos do gênero *Azospirillum,* que compreende atualmente 26 espécies (DSMZ, 2020).

Estudos têm mostrado que *Azospirillum brasilense* pode favorecer o desenvolvimento das plantas às quais se associa de diversas maneiras, entre elas pela produção de fitormônios que atuam diretamente no crescimento radicular, na fixação de nitrogênio atmosférico, na solubilização de fosfatos, no controle biológico de insetos e fitopatógenos e no aumento da resistência do vegetal aos estresses salino, hídrico e oxidativo (DÖBEREINER, 1979; DUCA et al., 2014; CEREZINI et al., 2016; FUKAMI et al., 2017, 2018a,b; HUNGRIA et al., 2018; RONDINA et al., 2020). Esses múltiplos mecanismos, tanto em *A. brasilense*, como em outras espécies, levaram à denominação dessas bactérias como promotoras do crescimento de plantas (BPCP).

Em 2009, após um extenso trabalho para seleção de estirpes realizado pela Embrapa Soja, foi lançado no mercado o primeiro inoculante brasileiro para milho (*Zea mays* L.) e trigo (*Triticum aestivum* L.), contendo as estirpes Ab-V5 e Ab-V6 de *Azospirillum brasilense*. Um ano após foram comrcializadas 300 mil doses e, nos anos seguintes, esse número cresceu consideravelmente atingindo, em 2019, o valor expressivo de 10,5 milhões de doses. Esse salto nas vendas pode ser atribuído aos excelentes resultados obtidos pela inoculação, ao aumento do rendimento das culturas a baixo custo e à recomendação de uso em outras culturas, como nas leguminosas e nas gramíneas forrageiras..

De forma geral, a obtenção de altos rendimentos pelas culturas pode ser limitada tanto pela disponibilidade de nutrientes quanto pela ocorrência de pragas e doenças. Algumas práticas são fundamentais para amenizar prejuízos e, consequentemente, contribuir para a fitossanidade, como por exemplo, o uso de cultivares resistentes, sementes livres de patógenos e o tratamento químico (MERTZ; HENNING; ZIMMER, 2009).

A manipulação genética para o desenvolvimento de cultivares resistentes é a maneira mais eficaz no controle de fitopatógenos, entretanto, ainda não foram desenvolvidas cultivares resistentes para a maioria das doenças. Atualmente, o uso de agrotóxicos como tratamento químico, seja nas sementes, no sulco, ou diretamente na planta representa a alternativa mais viável para o controle dessas doenças e redução de perdas (WORDELL FILHO et al., 2016). No Brasil, estima-se que 98% das sementes de soja (*Glycine max* (L.) Merr.) e de milho híbrido sejam tratadas com fungicidas e inseticidas (SPADOTTO et al., 2004; COTA et al., 2013; NUNES, 2016).

O manejo de doenças com o auxílio de agrotóxicos, aliado à inoculação de sementes com BPCPs podem ser considerados tecnologias muito importantes para garantir elevada produtividade do milho. Entretanto, recentemente estudos têm apontado incompatibilidade entre as bactérias presentes nos inoculantes com a prática de tratamento de sementes com agrotóxicos. A presença do agrotóxico pode prejudicar a viabilidade e o metabolismo da bactéria, causando prejuízos na contribuição e/ou produção de fitormônios e reduzindo os benefícios da inoculação sobre o desenvolvimendo das plantas.

Especificamente para o milho, ainda são poucas as informações sobre os efeitos da combinação do tratamento químico de sementes dessa cultura com as estirpes Ab-V5 e Ab-V6 presentes nos inoculantes, bem como sua influência sobre o desenvolvimento do vegetal. Um fator que limita estudos com essa abordagem é a ausência de um método que permita a recuperação de células de *A. brasilense* inoculadas em sementes de milho tratadas com agrotóxicos, a fim de se verificar a viabilidade após diferentes períodos de exposição. Um método semelhante é bastante consolidado para recuperação de *Bradyrhizobium* spp. inoculado em sementes de soja (*Glycine max* (L.) Merr), o que possibilita que mais infomações sobre a combatibilidade entre esses produtos sejam obtidas.

Devido à sua grande adaptabilidade e diversificada aplicabilidade, o milho ocupa, atualmente, o terceiro lugar entre os cereais mundialmente mais cultivados (AWIKA, 2011; SANTOS et al., 2019). No Brasil, seu cultivo é realizado em todos os 27 estados, com destaque para a região sul e estima-se que a safra 2020/21, deverá apresentar uma área total de 19,09 milhões de ha, com produção de 105,4 milhões de toneladas, e produtividade média de 5.525 kg ha^{-1} (CONAB, 2021).

Apesar da grande demanda nacional pelo milho (aproximadamente 72 milhões de toneladas em 2020/2021), o Brasil também é um importante exportador dessa *commodity* e ocupa o segundo lugar no *ranking* dos principais países exportadores (FIESP, 2019). Segundo dados da Conab (2021), na safra 2020/2021, espera-se que o volume de embarques seja de aproximadamente 35 milhões de toneladas.

Os objetivos desse trabalho foram: (*i*) caracterizar a situação mundial quanto ao denvolvimento e aplicação de inoculantes; (*ii*) caracterizar a eficácia das estirpes Ab-V5 e Ab-V6 de *A. brasilense* em estudos conduzidos no Brasil; (*iii*) desenvolver uma metodologia para recuperar células viáveis de *A. brasilense* após a inoculação de sementes de milho; (*iv*): avaliar o impacto de agrotóxicos utilizados no tratamento de sementes de milho para a sobrevivência de estirpes de *A. brasilense*, na promoção do crescimento de milho; *(v)*: evidenciar os principais resultados de estudos que investigaram os efeitos de fungicidas, inseticidas e herbicidas sobre inoculantes microbianos.

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CAPÍTULO 1

INOCULANTES MICROBIANOS: REVISANDO O PASSADO, DISCUTINDO O PRESENTE E PREVENDO UM FUTURO BRILHANTE PARA O USO DE BACTÉRIAS BENÉFICAS NA AGRICULTURA

INOCULANTES MICROBIANOS: REVISANDO O PASSADO, DISCUTINDO O PRESENTE E PREVENDO UM FUTURO BRILHANTE PARA O USO DE BACTÉRIAS BENÉFICAS NA AGRICULTURA

RESUMO

Mais de cem anos se passaram desde o desenvolvimento do primeiro inoculante microbiano para plantas. Atualmente, o uso de inoculantes microbianos na agricultura é globalmente adotado para diferentes culturas e inclui diferentes microrganismos. Nas últimas décadas, foram alcançados progressos impressionantes na produção, comercialização e uso de inoculantes. Atualmente, os agricultores são mais receptivos ao uso desse bioinsumo, principalmente devido à alta qualidade, visto que muitos deles contêm estirpes elite de múltiplos propósitos, melhorando os rendimentos das culturas a baixo custo em comparação com os fertilizantes químicos. No contexto de uma agricultura mais sustentável, os inoculantes microbianos também ajudam a mitigar os impactos ambientais causados pelos agrotóxicos. Os desafios consistem em realizar uma produção de inoculantes microbianos para uma ampla variedade de culturas e na expansão da área inoculada em todo o mundo, além da busca por soluções microbianas inovadoras em áreas sujeitas a episódios crescentes de estresse ambiental. Nesta revisão, exploramos o mercado mundial de inoculantes, mostrando quais bactérias são proeminentes como inoculantes em diferentes países e discutimos as principais estratégias de pesquisa que podem contribuir para melhorar o uso de inoculantes microbianos na agricultura.

Palavras-chave: Fixação biológica de nitrogênio, bactérias promotoras de crescimento de plantas, *Azospirillum*, inoculação, adubos químicos.

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MINI-REVIEW **Open Access**

Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture

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Abstract

More than one hundred years have passed since the development of the first microbial inoculant for plants. Nowadays, the use of microbial inoculants in agriculture is spread worldwide for different crops and carrying different microorganisms. In the last decades, impressive progress has been achieved in the production, commercialization and use of inoculants. Nowadays, farmers are more receptive to the use of inoculants mainly because high-quality products and multi-purpose elite strains are available at the market, improving yields at low cost in comparison to chemical fertilizers. In the context of a more sustainable agriculture, microbial inoculants also help to mitigate environmental impacts caused by agrochemicals. Challenges rely on the production of microbial inoculants for a broader range of crops, and the expansion of the inoculated area worldwide, in addition to the search for innovative microbial solutions in areas subjected to increasing episodes of environmental stresses. In this review, we explore the world market for inoculants, showing which bacteria are prominent as inoculants in different countries, and we discuss the main research strategies that might contribute to improve the use of microbial inoculants in agriculture.

Keywords: Biological nitrogen fixation, Plant-growth-promoting bacteria, *Azospirillum*, PGPB, PGPR, Inoculation, Rhizobia, Chemical fertilizers

Introduction

Humanity has always been concerned about food production to attend the increasing population and, for a long time, the solution was to expand agriculture to new areas. However, this scenario has changed in recent decades, first due to limitations of unexplored cultivable land, but also reinforced by the development of new technologies that allow higher yields, in addition to increasing environmental concerns, leading to agricultural practices aiming at achieving sustainable production. Therefore, although the global demand for food continues to increase, the concepts of agriculture sustainability, recovery of degraded areas, and mitigation of environmental impacts are gaining more respect (Canfield et al. 2010; Godfray et al. 2010). In this context, microbial inoculants—denominated as biofertilizers in some countries—have received increasing attention, gaining prominence and market scale in agriculture.

Inoculants are products that have in their composition living microorganisms capable of benefiting the development of different plant species. The most antique microorganisms used as inoculants are the "rhizobia", diazotrophic bacteria able to colonize the rhizosphere and establish nodules in the roots of their host plants, composed by several species of the Fabaceae family. The symbiosis legumes-rhizobia leads to the process of biological nitrogen fixation (BNF), which very often

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can fully supply the plant´s demands on N. Moreover, other diazotrophic bacteria, such as *Azospirillum*, establish less straight relationships with the host plant, but are also able to supply, at least partially, the plant's demands on N. Both *Azospirillum* and rhizobia, among other diazotrophic and non-diazotrophic bacteria are named as plant-growth-promoting bacteria (PGPB) or plant-growth-promoting rhizobacteria (PGPR), as they may benefit the plants by a variety of single or combined processes, including the production of phytohormones, siderophores, phosphate solubilization, induction of plant intrinsic systemic resistance to abiotic and biotic stresses, among others (Bhattacharyya and Jha 2012; Malusá and Vassilev 2014; Fukami et al. 2017, 2018a, b). Other microorganisms have also been increasingly used in agriculture for biological control of pests and diseases (Ciancio et al. 2016; Berg et al. 2017; Singh et al. 2017; Xiang et al. 2017), but this review will only deal with inoculants carrying strains that facilitate plant growth. Moreover, we will name all rhizobia and other bacteria carrying different mechanisms that promote plant growth as PGPB.

Currently, soybean (*Glycine max* (L.) Merr.) is the most inoculant-consuming crop worldwide, carrying bacteria belonging to the genus *Bradyrhizobium*. Brazil is probably the global leader in the use of inoculants for the soybean crop (Hungria and Mendes 2015; Okon et al. 2015; ANPII 2016) where approximately 78% of the copping area—nowadays 36.5 million hectares—is inoculated yearly (ANPII 2018). Additionally, inoculation of common beans (*Phaseolus vulgaris* L.), cowpea (*Vigna unguiculata* (L.) Walp.), maize (*Zea mays* L.) and co-inoculation of soybean and common bean with rhizobia and *Azospirillum* have also increased in Brazil (Hungria et al. 2010, 2015), so that the number of doses commercialized in the last years has impressively grown (Fig. 1). Other top countries in the use of inoculants are Argentina and India (Mazid and Khan 2014; Hungria and Mendes 2015; Okon et al. 2015; Sruthilaxmi and Babu 2017).

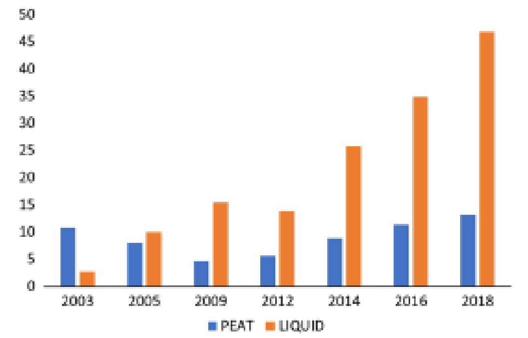

Fig. 1 Market of microbial inoculants in Brazil in the last 15 years (million doses)

However, there are limiting factors that restrict the use of inoculants in some areas. Biotic and abiotic stresses may affect the effectiveness of the product, making them inefficient in cases such as nutrient-poor or unbalanced soils, salinity, water stress, increasing temperatures, pests and diseases, among others (Bashan et al. 2014; Das et al. 2017; Khan et al. 2017; Thilakarathna and Raizada 2017; Samago et al. 2018). To circumvent these factors, several studies have been addressed to gain better knowledge on the intrinsic properties of PGPB, seeking at understanding their optimum growth conditions and interaction with the host plants (Flores-Félix et al. 2018; Goulart-Machado et al. 2018; Jiménez-Gómez et al. 2018). Efforts have also been applied to improve the efficiency of microorganisms already available and in the identification of new elite strains to be used as inoculants under unfavorable and stressful environmental conditions, such as areas frequently experiencing drought, soils with low nutrient availability or with salinity, among others (Benidire et al. 2017; Koskey et al. 2017; Youseif et al. 2017). There is an increasing number of studies aiming to isolate, identify and evaluate the capacity of plant-growth promotion of bacteria with a variety of plant species, with potential to be transformed into new microbial inoculants in a near future (Yanni et al. 2016; Koskey et al. 2017; Manasa et al. 2017; Muleta et al. 2017).

Another technology with increasing application relies on the use of mixed inoculants, aiming to promote plant growth by combining distinct mechanisms of different microorganisms. Mixed inoculants can provide excellent results and show the great potential of being increasingly used by the farmers (Juge et al. 2012; Hungria et al. 2013, 2015; Chibeba et al. 2015; Bulegon et al. 2017; Ferri et al. 2017).

The objective of this short review is to explore the current market of inoculants, highlighting what has been produced and marketed lately in several countries, and the impact on agricultural sustainability. We also explore new ideas, new objectives and new strategies that are needed to generate information for the development of new products, breaking down barriers needed to expand the use of microbial inoculants in agriculture.

Inoculant carriers

Since the beginning of the manufacturing of inoculants, the industry has been concerned about generating increasingly efficient products, at a low cost, whose handling attends to the needs and the quality required by farmers. An important aspect is the choice of the carrier for the microorganisms, which should, among other things, provide long cellular viability and be of easy application. In 1896, in the USA, the first inoculant commercially produced, "Nitragin" (Fig. 2), used gelatin, and later, nutrient medium was employed as carrier for bacterial cells. Due to the high mortality rate, these carriers were soon replaced by peat, which remained as the "gold" carrier until the end of the 1990s, when the scenario began to change (Fig. 2) (Williams 1984).

Peat is a solid material, consisting of organic soil naturally occurring in specific environments and formed after a long geological period. The choice of peat as carrier for inoculants is due to its richness in organic matter, which serves as an important source of nutrients for the microorganisms. The peaty matrix also provides physical protection to the microorganisms against soil adversities and allows better cell survival in conditions of water restriction and high temperatures (Hungria et al. 2000a, 2005). In the process of seed inoculation with peat it is essential to use adhesives to help to stick the peaty matrix to the seeds; for example, in Brazil the most popular adhesive is 10% sucrose solution (Hungria et al. 2000a). The peat-based inoculant must be packed in sterilized polyethylene or polypropylene bags, with thickness of 0.06–0.38 mm, which preserves moisture but guarantees gas exchange with the external medium (Hungria et al. 2005).

Concerns about the use of peat as inoculant carrier rely on the exploitation of peat bogs, which may cause serious environmental impacts, including the destruction of habitats and CO_2 emissions. In addition, in countries such as Brazil, where there are few peat bogs, importation of this material is required, increasing the production costs (Ribeiro et al. 2013). Due to these limitations, inoculants based on liquid formulations began to gain space, especially from the late 1990s onwards. In Brazil, the first liquid inoculant was approved by the Ministry of Agriculture for commercial used in 2000, and a decade later almost 80% of the inoculants sold in the country were in liquid formulations (Fig. 2); similar proportion is found in Argentina (ANPII 2018). Liquid inoculants consist of microbial cultures suspended in liquid medium rich in nutrients and cell protectors. They are easily handled and compatible with mechanized sowing, offering an advantage over solid inoculants at sowing. Another advantage is the easiness of sterilization, facilitating the absence of contaminants and, consequently, allowing higher cell concentration (Bashan et al. 2014; Cassán et al. 2015).

In addition to seed inoculation, liquid inoculants allow alternative application methods, such as in-furrow, and sprayed on soil or by "foliar" application (Campo et al. 2010; Fukami et al. 2016; Moretti et al. 2018). Alternative methods of application may be advantageous in some cases, for example, the inoculation in-furrow, to alleviate the impact of pesticides used for seed treatments in contact with the bacteria (Campo et al. 2010).

Other vehicles and methods for carrying microorganisms, such as agricultural and industrial residues, lyophilized bacteria and polymers for cell encapsulation, have been researched to develop more efficient and stable products. According to Bashan et al. (2014), industrial residues and agricultural by-products such as sugarcane bagasse, sawdust or brewery waste can be used as carriers for inoculation of microorganisms. However, the major limitation for the use of these raw materials is their poorly consistent composition, and often difficulties for sterilization.

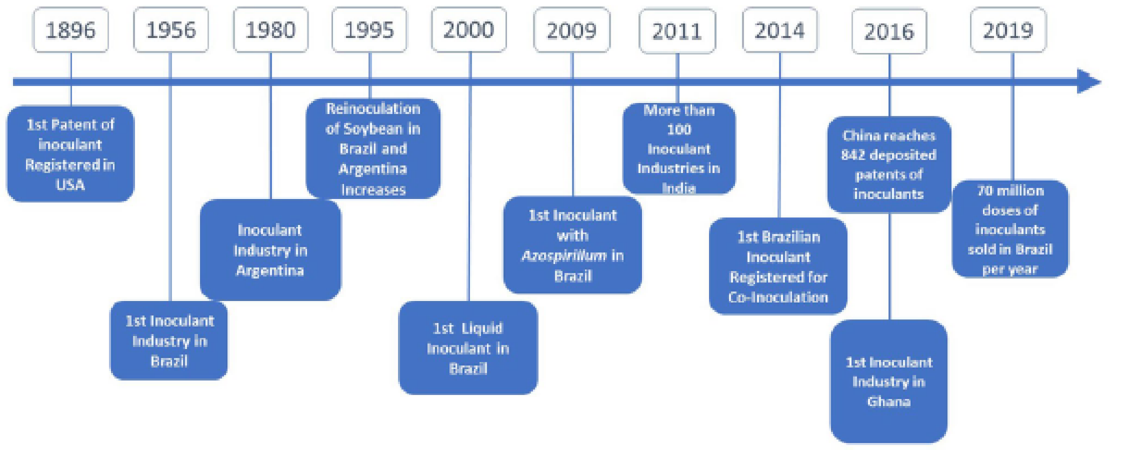

Fig. 2 Chronology of some important steps in the development of microbial inoculants

As one of the challenges for inoculant production is to maintain cell viability for long period, lyophilization and freezing of microbial cells have emerged as possibilities to overcome this problem. The process of lyophilization consists of removing the intracellular water, reducing the metabolic activity and increasing microbial lifetime. The dry cell culture must be mixed with a liquid or gel formulation at sowing. The great barrier to the commercial production of inoculants with lyophilized microorganisms is the high production cost because it requires specialized equipment and skilled labor (Williams 1984; Hungria et al. 2005). Besides, the time and conditions needed for cell recovering in liquid or gel formulations represent barriers for the adoption of inoculants by farmers, especially high large areas are cropped, such as for the soybean crop in South America.

The encapsulation of living microbial cells with polymers, such as alginate and polyacrylamide has also been increasingly mentioned. For the encapsulation, the liquid inoculant containing bacterial culture is mixed with an adjuvant polymer, capable of causing solidification. The most used method consists of mixing dropwise the microbial culture in a solution containing calcium chloride, resulting in solid beads with high cell concentration. The spheres are placed in contact with the seeds at sowing time and the bacteria are slowly released. These spheres are biodegradable and do not cause environmental impact. Encapsulation confers protection to the cells for high temperature and environmental stresses and are also easy to handle. Once again, the economic factors have always represented the main obstacle for scaling the industrial production (Bashan 1986; Bashan et al. 2002; Date 2001).

Great efforts have been applied by several industries to develop new products able to attend the new requirements of the market and compatible with new technologies. The trend for this next decade is to apply considerable investment in innovation, searching for new inoculant formulations to hit the ever growing market.

Inoculants containing mixes of bacteria

The great majority of the first manufactured inoculants contained only one species of microorganism, and in general one strain, the one with the best inoculation results for a particular crop. Exceptions included a maximum of two microorganisms "of the same type", for example, two *Bradyrhizobium* strains or species for soybean. The use of two strains in the same inoculant would increase the chances that at least one would nodulate and perform well with the legume. For example, in Brazil, the combination of two *Bradyrhizobium* strains for the soybean crop has been preferentially used by the farmers since the 1950s (Hungria et al. 1994; Hungria and Mendes 2015).

Particularly in the last decade, the use of inoculants containing microorganisms of "different type" has expanded. The idea is of combining strains or species acting in different microbial processes, so that the combined benefits of each one would result in higher benefits and, ultimately, yields. Examples of mixed inoculant are those combining microorganisms whose major processes are BNF (e.g. *Bradyrhizobium* spp., *Rhizobium* spp.) and phytohormone production (e.g. *Azospirillum* spp., *Pseudomonas* spp.), solubilization of phosphate (e.g. *Bacillus* spp.), or biological control (e.g. *Pseudomonas* spp., *Bacillus* spp.). If the microorganisms cannot be combined in a single product, they are manufactured separately and the bags containing each one are sold in the same package.

The application of mixed inoculants is usually called co-inoculation or mixed inoculation and it is currently possible to find co-inoculants for several crops in the market. The efficiency of co-inoculation is closely related to the appropriate selection of strains, the cellular concentration of each one, method of inoculation (applied to the seeds, leaf-spray, in-furrow), and to the plant genotype. Therefore, research is needed to generate knowledge aiming at the production of new formulations for commercial inoculants with mixed bacteria (Cassán et al. 2015), and on alternative methods of application of inoculants and microbial molecules (Campo et al. 2010; Fukami et al. 2016).

In Brazil, co-inoculation of *A. brasilense* with *Bradyrhizobium* spp. for the soybean crop and with *Rhizobium tropici* for the common beans was launched in 2014 and impressive increases in grain yield have been reported (Hungria et al. 2013, 2015; Souza and Ferreira 2017; Nogueira et al. 2018). Even in areas with high population of compatible rhizobia for both crops ($>10^4$ cells of compatible rhizobia/g soil), for the soybean crop single inoculation of *Bradyrhizobium* resulted in mean increases of 8.4% in grain yield compared with the naturalized population, whereas the co-inoculation with *A. brasilense* promoted an "upgrade" to 16.1%; for common beans, single inoculation with *R. tropici* increased yield by 8.3%, whereas the co-inoculation improved the yield by 19.6% (Hungria et al. 2013) (Table 1). Since them, other benefits attributed to the co-inoculation of soybean with *Bradyrhizobium* and *Azospirillum* in Brazil are the promotion of early nodulation (Chibeba et al. 2015), and increased tolerance to moderate water restriction (Cerezini et al. 2016; Silva et al. 2019).

In addition to *Azospirillum* spp., several other PGPB have been reported as successful in co-inoculation trials with soybean, as *Pseudomonas* sp. (Egamberdieva et al. 2017; Pawar et al. 2018), *Actinomyces* sp. (Nimnoi et al.

2014), *Bacillus* sp. (Atieno et al. 2012; Subramanian et al. 2014; Petkar et al. 2018). Improvements in yields have also been reported with the co-inoculation of rhizobia presenting different mechanisms of action. For example, Jesus et al. (2018) verified benefits by the co-inoculation of common bean with *R. tropici* CIAT 899, *Bradyrhizobium diazoefficiens* USDA 110 and *Bradyrhizobium elkanii* 29w; according to the authors, *Bradyrhizobium* spp. would improve the symbiosis efficiency of *Rhizobium*, resulting in greater number of nodules, biomass production and N accumulation. The suggested mechanism is that *Bradyrhizobium* sp. co-inoculated produces signaling molecules, such as nodulation factors (Nod factors) and surface polysaccharides that stimulate root nodulation by *R. tropici*, improving the efficiency of BNF.

Co-inoculation has also been shown to be efficient under several limiting conditions, such as in low phosphate soils. Generally, the BNF is compromised under these situations, but the co-inoculation with phosphate-solubilizing microorganisms can make it available for plant nutrition and, in the case of legumes, help to ensure the benefits of BNF (Jorquera et al. 2008; Morel et al. 2012; Shiri-Janagard et al. 2012; Korir et al. 2017). For example, Korir et al. (2017) evaluated the effects of co-inoculation in common beans grown in a soil with low P and observed that plants inoculated with *Rhizobium* strain IITA-PAU 987 and *Bacillus megaterium* increased nodulation, shoot dry weight and had 31% increase in BNF when compared with the single inoculation with *Rhizobium*.

Main inoculated crops

Soybean

Soybean is an annual herbaceous dicotyledonous, originally grown in the eastern region of Asia (Aliyev and Mirzoyev 2010). Until the nineteenth century, its cultivation remained restricted to the eastern countries, and spread to other continents, as America and Africa, only at the end of this period (Dall´Agnol et al. 2007; Aliyev and Mirzoyev 2010). Nowadays, the main soybean producers are the USA, Brazil, and Argentina.

Soybean is probably the most successful example of crop benefiting from the application of microbial inoculants, more specifically, carrying *Bradyrhizobium* spp. strains. South American countries lead soybean inoculation. In contrast, in the USA, estimates are that only 15% of the area cropped with soybean has been inoculated, what might be related to the low cost of N-fertilizer marketed in the country (Chang et al. 2015). The low cost of N-fertilizer may also have implied in lower interest in innovation of technologies updated with new agricultural practices.

The Brazilian research for the production and commercialization of inoculants is very advanced and the country has one of the most complete legislation in this area. Common resolutions for inoculants commercialization were defined in 1998 for the Mercosur, the common market including Brazil, Argentina, Uruguay and Paraguay. Following, in Brazil, a legislation of 2004 included definitions and norms on specifications, guarantees, registrations, packaging and labeling of inoculants, as well as the list of the microorganisms that could be used in commercial inoculants in the country; the document was updated in 2011 (MAPA 2004, 2011). Nowadays, four strains of *Bradyrhizobium* are authorized for the production of soybean inoculants in the country (*Bradyrhizobium japonicum* SEMIA 5079 (=CPAC 15), *B. diazoefficiens* SEMIA 5080 (=CPAC 7), *B. elkanii* SEMIA 5019 (=29w) and SEMIA 587). The legislation still establishes a minimum concentration of viable cells (1×10^9 viable cells/g or mL) of the inoculant until the expiration date, which must be at least 6 months, and void of contaminants at the 1×10^{-5} dilution (Hungria et al. 2010; MAPA 2011). The technical recommendation in Brazil indicates a dose that allows at least 1.2 million viable cells/seed to guarantee a successful nodulation (Hungria et al. 2017; Hungria and Nogueira 2019). The credibility of the inoculant market in Brazil relies on strict legal regulation. Interestingly, the legislation was created based mainly on the Australian legislation, where nowadays the regulation relies on an agreement between partners, as a voluntary control (Bullard et al. 2005; AIRG 2010).

In Brazil, the inoculation of soybean with elite *Bradyrhizobium* spp. strains can fully supply the crop's demand on N, dismissing the use of N-fertilizers. Probably as a result of breeding for BNF, the symbiosis with soybean is very sensitive to N-fertilizers, drastically reducing nodulation (Hungria et al. 2007; Hungria and Mendes 2015). Soybean cropping without any N-fertilizer has generated an annual economy that today is estimated at about 20 billion dollars.

In Brazil, Argentina and in other South American countries, successful results have been achieved with the re-inoculation of soybean, i.e., the yearly inoculation even in soils presenting well-established compatible rhizobial population from previous inoculations (Hungria et al. 2001; Hungria and Mendes 2015). This practice led to the commercialization of over 70 million doses of inoculants for soybean in Brazil in the last crop season. Estimates in Brazil are that re-inoculation increases soybean grain yield by 8% in average (Hungria and Mendes 2015) and by 6.8% (Leggett et al. 2017) to 14% (Hungria et al. 2016) in Argentina. In the USA, re-inoculation is traditionally not recommended, based on results from a former study

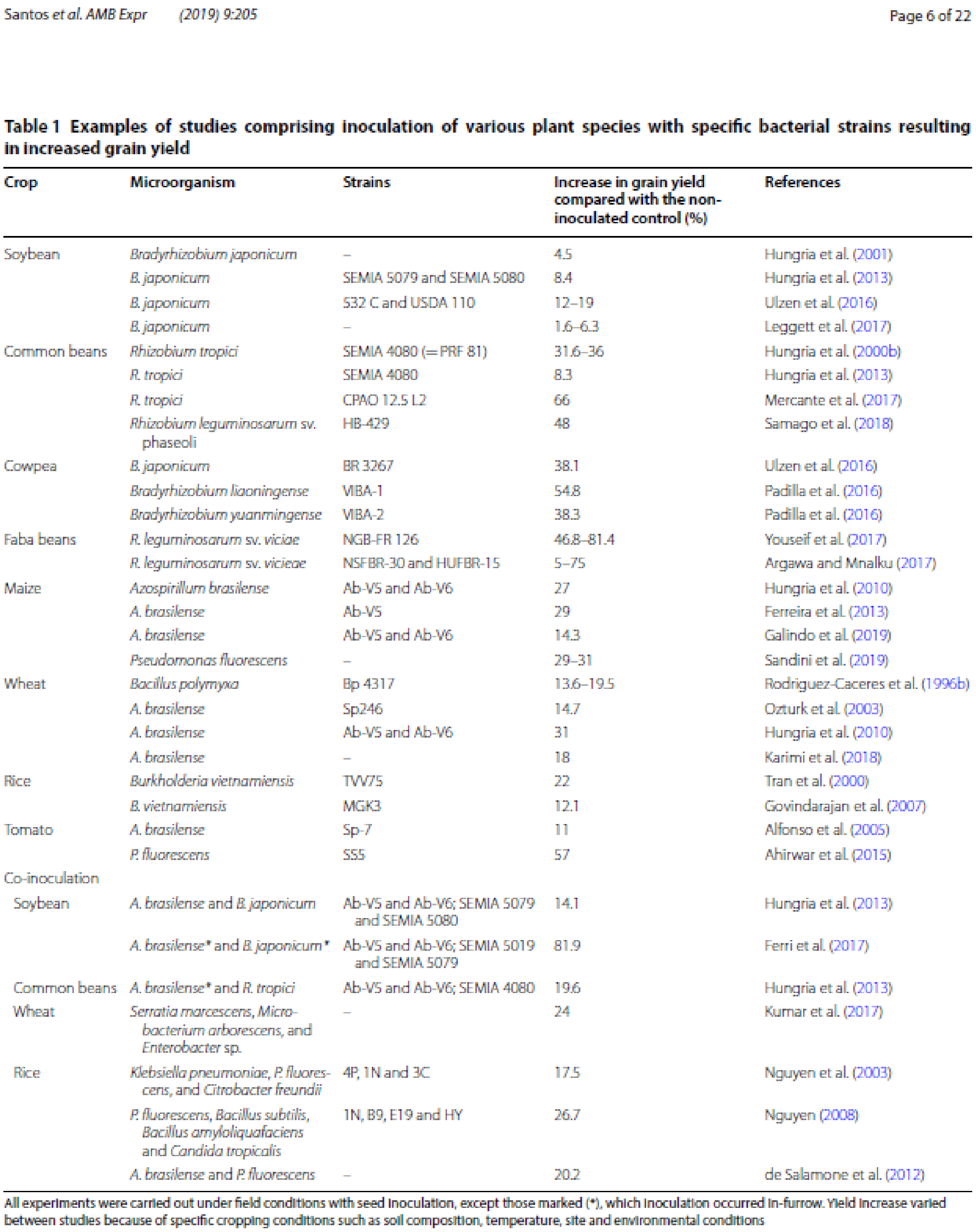

Table 1 Examples of studies comprising inoculation of various plant species with specific bacterial strains resulting in increased grain yield

Crop	Microorganism	Strains	Increase in grain yield compared with the non-inoculated control (%)	References
Soybean	*Bradyrhizobium japonicum*	–	4.5	Hungria et al. (2001)
	B. japonicum	SEMIA 5079 and SEMIA 5080	8.4	Hungria et al. (2013)
	B. japonicum	532 C and USDA 110	12–19	Ulzen et al. (2016)
	B. japonicum	–	1.6–6.3	Leggett et al. (2017)
Common beans	*Rhizobium tropici*	SEMIA 4080 (= PRF 81)	31.6–36	Hungria et al. (2000b)
	R. tropici	SEMIA 4080	8.3	Hungria et al. (2013)
	R. tropici	CPAO 12.5 L2	66	Mercante et al. (2017)
	Rhizobium leguminosarum sv. phaseoli	HB-429	48	Samago et al. (2018)
Cowpea	*B. japonicum*	BR 3267	38.1	Ulzen et al. (2016)
	Bradyrhizobium liaoningense	VIBA-1	54.8	Padilla et al. (2016)
	Bradyrhizobium yuanmingense	VIBA-2	38.3	Padilla et al. (2016)
Faba beans	*R. leguminosarum sv. viciae*	NGB-FR 126	46.8–81.4	Youseif et al. (2017)
	R. leguminosarum sv. vicieae	NSFBR-30 and HUFBR-15	5–75	Argawa and Mnalku (2017)
Maize	*Azospirillum brasilense*	Ab-V5 and Ab-V6	27	Hungria et al. (2010)
	A. brasilense	Ab-V5	29	Ferreira et al. (2013)
	A. brasilense	Ab-V5 and Ab-V6	14.3	Galindo et al. (2019)
	Pseudomonas fluorescens	–	29–31	Sandini et al. (2019)
Wheat	*Bacillus polymyxa*	Bp 4317	13.6–19.5	Rodriguez-Caceres et al. (1996b)
	A. brasilense	Sp246	14.7	Ozturk et al. (2003)
	A. brasilense	Ab-V5 and Ab-V6	31	Hungria et al. (2010)
	A. brasilense	–	18	Karimi et al. (2018)
Rice	*Burkholderia vietnamiensis*	TVV75	22	Tran et al. (2000)
	B. vietnamiensis	MGK3	12.1	Govindarajan et al. (2007)
Tomato	*A. brasilense*	Sp-7	11	Alfonso et al. (2005)
	P. fluorescens	SS5	57	Ahirwar et al. (2015)
Co-inoculation				
Soybean	*A. brasilense* and *B. japonicum*	Ab-V5 and Ab-V6; SEMIA 5079 and SEMIA 5080	14.1	Hungria et al. (2013)
	*A. brasilense** and *B. japonicum**	Ab-V5 and Ab-V6; SEMIA 5019 and SEMIA 5079	81.9	Ferri et al. (2017)
Common beans	*A. brasilense** and *R. tropici*	Ab-V5 and Ab-V6; SEMIA 4080	19.6	Hungria et al. (2013)
Wheat	*Serratia marcescens*, *Microbacterium arborescens*, and *Enterobacter* sp.	–	24	Kumar et al. (2017)
Rice	*Klebsiella pneumoniae*, *P. fluorescens*, and *Citrobacter freundii*	4P, 1N and 3C	17.5	Nguyen et al. (2003)
	P. fluorescens, *Bacillus subtilis*, *Bacillus amyloliquafaciens* and *Candida tropicalis*	1N, B9, E19 and HY	26.7	Nguyen (2008)
	A. brasilense and *P. fluorescens*	–	20.2	de Salamone et al. (2012)

All experiments were carried out under field conditions with seed inoculation, except those marked (*), which inoculation occurred in-furrow. Yield increase varied between studies because of specific cropping conditions such as soil composition, temperature, site and environmental conditions showing that rhizobial populations as low as 10 cells/g would inhibit the nodule formation by inoculant strains (Thies et al. 1991a, 1995). However, mean yield increases due to inoculation considering areas of traditional soybean cropping have been recently estimated at 1.67% (Leggett et al. 2017), but could probably be higher if high N-fertilizer levels were not applied to the crops comprising the soybean agricultural systems (Chang et al. 2015). Amazingly, even the most recent studies on the quantification of soybean BNF in the USA take into consideration a large number of sites, soil fertility, and application of mineral N, but not the re-inoculation component (Córdova et al. 2019). Certainly, the annual re-inoculation is responsible for the high contribution of BNF to the soybean N nutrition in Brazil, with values as high as 94% of the aboveground N accumulation (Hungria et al. 2006), while in the USA these values range from 23 to 65% (Córdova et al. 2019).

The Sub-Saharan Africa (SSA) region has developed, over the years, strategies for the use of beneficial microorganisms in soybean adapted to local environment and social characteristics. As consequence of the lack of local production and difficulties in the importation of inoculants in the 1970s, the International Institute of Tropical Agriculture (IITA) launched a breeding program aiming at developing high-yielding tropical soybean varieties capable of nodulating with indigenous rhizobial strains. These new varieties were named "TGx" (tropical *Glycine* cross) or "promiscuous" soybeans (Kueneman et al. 1984; Pulver et al. 1985), and contributed to the expansion of soybean production in the SSA.

Because the usually acidic, saline, and low organic matter of the SSA soils, the average soybean yield is usually well below the world average (Thuita et al. 2012; Muleta et al. 2017). Therefore, in addition to the soybean genetic breeding, further studies have been carried out aiming at increasing yields. For example, in Ethiopia, Muleta et al. (2017) searched for acid-tolerant rhizobia as strategy to increase soybean performance. A local isolate was able to improve soybean yield, indicating that search for indigenous or naturalized elite isolates might represent an interesting strategy to be adopted in other African countries. Impressive yield increases have also been observed by combining application of P-fertilizer and rhizobial inoculant in Nigeria (Ronner et al. 2016), and along with other studies suggest that P is probably the main limiting factor to the BNF in Africa (Vanlauwe et al. 2019).

In Mozambique, the majority of soybean cropping was with promiscuous varieties without inoculation; however, due to the increased demand on exportation of grains and poultry industry, the cultivation of non-promiscuous and more-productive cultivars associated with inoculation has increased (Dias and Amane 2011). As the agroclimatic conditions of the soybean production areas in Mozambique are similar to the main areas of soybean cultivation in the Brazilian savanna, Chibeba et al. (2018) evaluated and confirmed that elite strains identified in Brazil could have a successful performance in Mozambique with non-promiscuous soybean genotypes. The feasibility of transferring inoculation technologies between countries is of outstanding importance, as it can accelerate the establishment of sustainable cropping systems, saving time, labor and money. However, it is always desirable to search for indigenous or adapted strains, and promising local soybean strains have been identified in Mozambique (Chibeba et al. 2017), in a near future, their performance should be compared with the imported strains under field conditions.

Common beans

Similar to soybeans, common beans (*Phaseolus vulgaris* L.) are cropped worldwide, representing the most important source of protein in several countries, especially in South and Central America and Africa (Hungria et al. 2000b, 2013; Ribeiro et al. 2013). Although Brazil is one of the main producers (3.17 million hectares in the 2017/2018 crop season) and consumer of common beans worldwide, grain yields are usually low, bellow 1000 kg/ha (Hungria et al. 2007; CONAB 2019). Therefore, many strategies have been considered to improve yield, concomitantly to the tolerance to environmental stresses, at low cost.

Studies carried out in Brazil identified two strains of the "*R. tropici* group" for common bean that show high BNF rates, competitiveness, tolerance to environmental stresses and genetic stability (Hungria et al. 2000a, 2003; Mostasso et al. 2002). The strains PRF 81 (=SEMIA 4080) of *R. freirei* and H 12 (=SEMIA 4088) of *R. tropici* have been used in commercial inoculants in Brazil since 1998 and 2004, respectively, in addition to *R. tropici* CIAT 899, originally isolated in Colombia by Dr. Peter H. Graham (Hungria et al. 2000a, 2003; Gomes et al. 2015). Interestingly, CIAT 899 has been recognized as an outstanding strain in several countries (Gomes et al. 2015; Vanlauwe et al. 2019).

The use of inoculants for common bean favors yields, but there are reports indicating that BNF might not replace N-fertilizers completely, especially in soils where the N concentration is very low. Studies suggest that the application of 15 or 20 kg N/ha along with inoculation at sowing might improve grain yield (Soares et al. 2016), but higher doses of N at sowing may lead to reduced nodulation (Hungria et al. 2003). Noteworthy, Mercante et al. (2017), in a series of field trials performed in the Brazilian Cerrados verified that, in comparison with the indigenous population, the mean increase in grain yield by inoculating *R. tropici* CIAT 899 was of 410 kg/ha, but decreased to 365 kg/ha with the application of 20 kg of N/ha at sowing; a new identified elite strain resulted in outstanding mean increases of 665 kg/ha in grain production (Table 1).

The African continent also stands out in the production and consumption of common beans. Estimates are that 25% of the total world area cropped with common beans are in Africa, where the legume is part of the diet of more than 100 million people (Aserse et al. 2012; Beebe et al. 2013), with Tanzania, Kenya, Uganda and South Africa been the main producers (USDA 2012). Similar to South America, African researchers are studying different ways of increasing common bean yield by using BNF, especially in situations where the efficiency of nodulation by *Rhizobium* is compromised, such as dry conditions, low P concentrations, soil salinity and high temperatures (Yanni et al. 2016; Samago et al. 2018). In order to identify rhizobia capable of tolerating drought and salinity stresses, Yanni et al. (2016) selected indigenous strains in the eastern and western regions of the Nile delta, and identified elite strains with good performance under saline and water stress conditions, promising for the use as inoculants (Kanonge-Mafaune et al. 2018).

The approach of selecting adapted indigenous strains with high capacity of BNF was also investigated by Koskey et al. (2017) in soils of low fertility in Kenya. Regarding the symbiotic efficiency, four indigenous isolates showed good symbiotic performance, one being able to increase grain yield by 30% in comparison to the commercial inoculum for beans, Biofix (strains not informed). The importance of P for the symbiotic performance of common bean was highlighted under field conditions in Nigeria (Ronner et al. 2016) and Ethiopia (Samago et al. 2018).

Cowpea

Originated from the African continent, cowpea (*Vigna unguiculata* L. Walp.) is the major legume cropped in many African countries, responsible for more than 95% of the world's production (Silva et al. 2016). In Brazil, cowpea was introduced in the sixteenth century and has been cultivated mainly in the North and Northeast regions. Despite the still modest yield, Brazil has exported cowpea grains to some countries such as India, Egypt and Pakistan (Silva et al. 2016).

Cowpea is usually tolerant to high temperatures, low soil fertility and water restriction; grain yield can be limited by N availability, which can be supplied by BNF. Interestingly, African countries with climate and humidity conditions similar to the North and Northeast of Brazil have tested and observed positive responses to inoculation with elite *Bradyrhizobium* strains from Brazil. Boddey et al. (2016) and Ulzen et al. (2016) observed significant increases in nodulation and yield of cowpea inoculated with Brazilian rhizobia in northern Mozambique and northern Ghana.

Other indigenous microorganisms have also been identified, selected and proved to increase cowpea yield. A study carried out in a saline soil in Cuba demonstrated the efficacy of two indigenous strains (*Bradyrhizobium liaoningense* VIBA-1 and *Bradyrhizobium yuanmingense* VIBA-2) (Padilla et al. 2016) (Table 1). In another study, in Bangladesh, one strain isolated from cowpea nodules was identified as *Rhizobium* sp. SOY7 and presented excellent results of nodulation and plant growth, when compared with the non-inoculated control (Nushair et al. 2017).

Faba beans

Used in Chinese cooking for at least 5000 years, the origin of faba beans (*Vicia faba* L.) is still controversial (Duc 1997). Currently, the crop is produced and consumed in several countries, due to its adaptation to various climatic zones. The main producers are China, Italy, Spain, United Kingdom, Egypt, Ethiopia, Morocco, Russia, Mexico and Brazil (Duc et al. 2010; Lavania et al. 2015). However, there has been a considerable decline in the cropped area worldwide, mainly due to susceptibility to environmental stresses, affecting yield stability (Rubiales 2010).

In relation to the capacity of BNF, many soils favor the development of compatible rhizobial strains (Köpke and Nemecek 2010). The identification of rhizobia from nodules of faba beans indicate that the most common species are *Rhizobium leguminosarum* bv. viciae, *Rhizobium fabae*, *Rhizobium laguerreae* and *Rhizobium anhuiense* (Mutch and Young 2004; Tian et al. 2008; Saïdi et al. 2014; Zhang et al. 2015). Because of the high population of rhizobia in areas cropped with the legume for a long time, inoculation is usually not adopted. However, in regions where faba beans are not intensively cropped, or under stressful conditions, inoculation can benefit plant development (Köpke and Nemecek 2010; Youseif et al. 2017).

Faba beans are one of the most consumed grains in Egypt. Despite the predominantly low-fertility soils, inoculation is usually not performed and grain production is low, not attending the country's demand. However, the potential of response to inoculation has been demonstrated in some studies, e.g. Youseif et al. (2017) evaluated 17 indigenous rhizobial strains from different regions of Egypt, and observed that seed inoculation increased grain yield (Table 1) and N accumulation, reaching up to 155 kg ha of N in grains.

In saline soils in Morocco, Benidire et al. (2017) reported two indigenous strains of *R. leguminosarum*

(RhOF34 and RhOF125) that induced plant protection against salinity, leading to increases in nodulation, plant biomass and N content, confirming that indigenous species may have excellent results when inoculated in fava beans.

Other legumes

Legumes are generally part of the food base of people and animals throughout the world. In addition to soybeans and various types of beans, other crops are also important sources of protein and nutrients and serve as raw materials for many industrialized products. Therefore, raising the yield of these crops under a variety of environments, by means of inoculation with elite rhizobial strains has been the subject of several studies in several countries.

In Brazil, *Bradyrhizobium* sp. strain SEMIA 6144, originally from Africa, has been used in commercial inoculants for peanut (*Arachis hypogaea*); however, inoculation is not a common practice for this crop in the country, attributed to the lack of response, due to the high population of indigenous rhizobia. Indeed, peanut is a very promiscuous species capable of nodulating with a broad range of soil rhizobia (Thies et al. 1991b). However, an efficient contribution of the BNF may require specific elite strains, adapted to local biotic and abiotic conditions and may vary with the plant genotype. For example, Marcondes et al. (2010) evaluated the BNF efficiency of isolates from two peanut varieties (IAC 886 Runner and IAC Tatu ST) and verified that the bacteria performance varied with the plant genotype.

In 2017 the first inoculant was produced for peanuts in Africa, 1 year after the establishment of the first industrial plant for inoculant production in Ghana, in a partnership with Brazil (Fig. 2). Although it is still in the testing phase, the results are promising and peanut growers are expected to benefit from inoculation in the coming years.

Chickpea (*Cicer arietinum*) is a highly nutritive legume cropped mainly in India, but also in more than fifty other countries (Jukanti et al. 2012). Bacteria of the genus *Mesorhizobium* sp. are commonly found in association with chickpea (Laranjo et al. 2014) and *Mesorhizobium ciceri* has already been indicated for the production of inoculants. In Australia, *M. ciceri* strain CC1192 has been used in inoculants since the 1970s (Bullard et al. 2005). Besides, several studies have been carried out to identify indigenous strains capable of nodulating and promoting chickpea growth, even in unfavorable environments, such as low-fertility soils (Tena et al. 2016; Pandey et al. 2018).

Guar (*Cyamopsis tetragonolobus* L.) is a legume that has gained prominence in global agriculture due to several industrial uses, as their seeds are rich in galactomannan gum, which can be used as lubricant, binder, thickener and emulsifier. It is cultivated in several semi-arid regions such as in India, Pakistan and the United States (Ibrahim et al. 2016; Thapa et al. 2018). Similar to other legumes, guar has the potential to associate with rhizobia, but the process of nodulation with rhizobia is still not well known (Abidi et al. 2015); therefore, studies have been performed to identify elite rhizobial strains (Ibrahim et al. 2016; Khandelwal and Sindhu 2012). Thapa et al. (2018) evaluated two guar varieties inoculated with two rhizobial inoculants, one composed by a complex mixture of *Rhizobium* and the other carrying *Rhizobium* USDA 3385, on two soils of different textures, and promising results were found, as abundant nodulation, incentivizing further experiments.

An increasing number of yields increase have been reported for important crops such as soybeans, common beans and chickpeas inoculated with elite rhizobial strains, leading to interest in using microbial inoculants for several other legumes. However, it has also increased the interest for the use of other plant-growth promoting bacteria in non-legumes.

Maize

Maize (*Zea mays* L.) is a native grass from Central America (Doebley 1990a, b), and currently the third most cultivated cereal in the world. The interest in maize production is due to its versatility and broad use, ranging from human and animal feed to the production of biofuel, and also as an input in the manufacture of many products (Awika 2011). The main producers and consumers are the USA and China, followed by Brazil (DERAL 2019).

Maize can associate with PGPB, particularly those belonging to the genus *Azospirillum*, which are currently used as inoculants for this crop worldwide. Mexico was one of the first countries to commercialize inoculants for maize carrying *Azospirillum* in 2002 (Reis 2007), followed by Argentina.

Brazil has a long tradition in studies with *Azospirillum*, carried initially by Dr. Johanna Döbereiner. She described the capacity of *Azospirillum*, originally named as *Spirillum*, to perform BNF when associated with grasses. In 1978 the species *Spirillum lipoferum*, initially described by Beijerinck (1925), was reclassified as *Azospirillum*, with the prefix "azo" added as a reference to the term "azote," nomenclature given by Lavoisier to nitrogen. At that time, the genus comprised two species, *Azospirillum lipoferum* and *Azospirillum brasilense* (Tarrand et al. 1978). Other species of *Azospirillum* were described in the following years, so that in 2019 the genus comprises 21 species (DSMZ 2019).

However, it was only in 2009 that the first commercial strains of *A. brasilense*, Ab-V5 and Ab-V6, were released for the use in commercial inoculants for maize and wheat (*Triticum aestivum* L.) in Brazil (Hungria et al. 2010; MAPA 2011). In maize, these strains resulted in increases in grain yield that reached 27%, compared with the non-inoculated control (Hungria et al. 2010) (Table 1). Since the release of the first commercial inoculant for grasses in Brazil, in 2009 (Fig. 2), the number of sold doses of inoculants carrying *A. brasilense* has grown significantly, reaching about 7 million doses in the 2017/18 crop season. In Argentina, the market of *Azospirillum* has started before Brazil, with the commercial strain *A. brasilense* Az39 selected in the 1980s and able to increase maize and wheat yields from 13 to 33% (Cassán et al. 2015; Cassán and Diaz-Zorita 2016).

In addition to its ability for BNF, numerous studies have demonstrated other properties of *Azospirillum*, the most important being the capacity for synthesizing phytohormones. Many of these molecules are related to root development, positively influencing their growth, resulting in greater absorption of nutrients and water from soil (Bashan and De-Bashan 2010; Ardakani and Mafakheri 2011; Fukami et al. 2017, 2018a, b). Therefore, grasses associated with *Azospirillum* present root structure capable of absorbing larger amounts of nutrients and water (Bashan and De-Bashan 2010). Auxins (Fallik et al. 1989; Fukami et al. 2017), gibberellins (Janzen et al. 1992; Cohen et al. 2009), ethylene (Perrig et al. 2007), cytokinins (Strzelczyk et al. 1994; Abbasi et al. 2015) and salicylic acid (Perrig et al. 2007; Cohen et al. 2009; Fukami et al. 2017) are the most commonly cited molecules.

Turan et al. (2012) emphasized the capacity of P solubilization by some strains of *Azospirillum*, increasing P availability in the soil and yields of wheat. Some strains of *Azospirillum* may also attenuate damages caused by abiotic stress, such as salinity and drought, as well as biotic stresses, like plant resistance against pathogens (Bashan and De-Bashan 2010; Fukami et al. 2018a).

Despite the benefits of *Azospirillum* in cereals, the bacterium is not able to supply all N demand, requiring the application of complementary doses of N. However, the amount of N-fertilizer to achieve high yields can be reduced by 25 to 50% (Hungria et al. 2010; Piccinin et al. 2013; Fukami et al. 2016).

Although *Azospirillum* is mainly inoculated on the seeds due to easiness and low doses (Cassán et al. 2015), the seed treatment with pesticides is potentially harmful and may impair the survival and metabolism of the inoculated cells. To overcome such problem, alternative methods of inoculation via foliar, in-furrow or soil spraying can be used. Fukami et al. (2016) evaluated the responses of maize inoculated with *Azospirillum* in-furrow, via soil spraying at sowing or via leaf spraying after seedlings had emerged, in comparison seed inoculation. Positive results were obtained with both alternative methods of inoculation, but higher doses were required than inoculation via seeds.

Besides *Azospirillum*, other groups of PGPB have been studied in inoculation of maize, such as *Pseudomonas* spp. (Burr et al. 1978; Ahirwar et al. 2015; Thirumal et al. 2017; Sandini et al. 2019). *Pseudomonas* are able to produce siderophores, which are molecules capable of capturing insoluble iron from the environment (Fe^{3+}), and convert it to a soluble form (Fe^{2+}) available for plants (Sharma and Johri 2003; Sah et al. 2017). Considering that iron is essential for metabolism and consequently, for plant development, the siderophores-producing microorganisms can positively improve plant development in Fe-deficient environments.

The production of siderophores by *P. aeruginosa* strains RSP5 and RSP8 was demonstrated in iron sufficient and iron-deficient soil (Sah et al. 2017). The strain RSP5 produced more siderophores in both soils and improved the Fe uptake by maize, in addition to increases in shoot and root length, number of spikes and number of grains. However, we must emphasize that many PGPB may also be highly pathogenic to humans, animals and plants. Therefore, it is critical to evaluate the non-pathogenicity of the strains before thinking about any use as inoculant and, certainly, *P. aeruginosa* is not a proper candidate for a commercial inoculant.

The use of *Bacillus* strains as inoculants is also increasing, in replacement to fertilizers. In Brazil, strains have been selected that improve P mobilization, by mechanisms as phytohormones production and P solubilization, this last one attribute to acid production by the bacteria (de Abreu et al. 2017). In Brazil, elite strains of *Bacillus* proved to improve P uptake production of grasses (Ribeiro et al. 2018), and the first commercial inoculant carrying P-solubilizing bacteria (*Bacillus subtilis* and *B. megaterium*) was released in 2019, with great acceptance by the farmers.

Wheat

Wheat is a cereal of global importance for human and animal feeding and can also benefit from inoculation with *A. brasilense* (Bashan et al. 2004; Hungria et al. 2010). In the 1980s an important study was carried out in Mexico on the inoculation of wheat with *Azospirillum*. The concentration of the inoculant was 3–5 × 10^8 CFU/g and the dose applied of 15 g/kg seed. Inoculation caused significant increases in yield, from 23 to 63% in 1986, and from 24 to 43% in 1987. The best results were obtained with strain Cd and with a local *A. brasilense* strain isolated from the rhizosphere of *Brachiaria mutica* (UAP-55) (Caballero-Mellado et al. 1992).

In the following decade, in Argentina, many studies were carried out with inoculation of *Azospirillum*. In 1992–1993 two experiments were carried out with inoculation of strains Az39 and Cd on wheat under greenhouse conditions using soil from a semiarid region of Argentina. Az39 and Cd strains increased the grain yield by 30% and 16%, respectively, and both increased the root dry weight compared with the non-inoculated control (Rodriguez-Caceres et al. 1996a). Nowadays, Az39 is the major strain used in commercial inoculants in Argentina (Okon et al. 2015).

In Brazil, Hungria et al. (2010) observed 13 to 18% increase in grain yield of wheat inoculated with *A. brasilense* Ab-V1, Ab-V5, Ab-V6 and Ab-V8 strains. When the strains Ab-V5 and Ab-V6 were combined, wheat yields increased by 31%; therefore, inoculant industries have mixed both strains in wheat inoculants (Hungria et al. 2010) (Table 1).

Further beneficial action of *A. brasilense* has been reported on wheat, such as the photo-protection of photosynthetic pigments and increase of proton efflux of roots, positively affecting plant development (Bashan et al. 1989, 2005).

Successful wheat inoculation with *Azospirillum* has also being reported in Israel (Kapulnik et al. 1983, 1985), England (Harris et al. 1989), Egypt (El-Lattief 2012), and Pakistan (Zaheer et al. 2019). Unfortunately, despite numerous studies proving the benefits of wheat inoculation, this practice is poorly adopted, especially in the major wheat-producing countries such as European Union, Russia, China, India and the United States.

Rice

The origin of rice (*Oryza sativa*) is estimated at least 130 million years ago in Asia and has spread over the years all over the planet (Khush 1997), representing about 11% of the global cropped area. This cereal represents the primary source of food for more than one-third of the world's population; unlike other crops, rice is consumed almost exclusively by humans (Khush 1997; Singh et al. 2018).

More than 90% of the world's rice is grown and consumed in Asia, where it accounts for 35 to 60% of the calories consumed by 3 billion people, 60% of the worlds' population (Khush 1997; Seck et al. 2012; Singh et al. 2018). The main producers are China, India, Indonesia and Bangladesh, with the production of 145.5; 103.5; 36.3 and 34.6 million tons, respectively (Gadal et al. 2019).

Similar to the grasses earlier mentioned, rice can also benefit from the inoculation with PGPB. Although rice is typically grown in wetland, upland cropping is very important in several countries. In wetland, rice can be associated with aerobic and anaerobic PGPB (Choudhury and Kennedy 2004). Many bacterial species have been evaluated over the years, single or associated, for growth promotion of rice, e.g. *A. lipoferum* (Watanabe and Lin 1984; Mirza et al. 2000), *A. brasilense* (de Salamone et al. 2012; Zhang et al. 2017) *Pseudomonas* spp. (Watanabe and Lin 1984; de Salamone et al. 2012; Zhang et al. 2017), *Herbaspirillum* spp. (Baldani et al. 2000; Mirza et al. 2000), *Burkholderia* spp. (Baldani et al. 2000; Tran et al. 2000; Govindarajan et al. 2007), *Bradyrhizobium* sp. (Greetatorn et al. 2019).

One of the most important studies related to inoculants for rice was carried out in Vietnam from 1999 to 2001 (Nguyen et al. 2003) and resulted in a commercial inoculant named "Biogro". Three bacterial strains isolated from soils cropped with rice were selected and their inoculation promoted increase in grain yield compared with the non-inoculated control, reaching yields of 6.7; 6.0 and 6.2 t/ha in 1999, 2000 and 2001, respectively, when 111 kg/ha of biofertilizer were applied; the overall mean increase over the non-inoculated control was of 15% (728 kg/ha), ranging from 8.3 to 30.7%. (Nguyen et al. 2017). Similar results were obtained 1 year later in Australia, using the same mix of bacteria (Williams and Kennedy 2002).

Before 2005, the strains in "Biogro" were *Klebsiella pneumoniae* (4P), *Pseudomonas fluorescens* (1N) and *Citrobacter freundii* (3C) (Kecskes et al. 2008). From 2005 on, the inoculant was reformulated with the strains *P. fluorescens* (1N), *B. subtilis* (B9), *Bacillus amyloliquafaciens* (E19) and a soil yeast, *Candida tropicalis* (HY) (Nguyen et al. 2017). In addition to BNF, the pool of microorganisms also improved the P mobilization from soil. In field trials the new inoculant applied at a rate of 50 kg/ha promoted grain yield of 6.91 t/ha (Nguyen 2008; Nguyen et al. 2017) (Table 1). This inoculant was also efficient in rice grown on a degraded soil in the south of Vietnam (Phan and Tran 2008).

Sugarcane

An economically important Poaceae is sugarcane. Belonging to the genus *Saccharum*, it is native from the tropical region of South and Southeast Asia (Mukherjee 1957). After many taxonomic revisions that occurred mainly during the twenty ninth century, currently the genus *Saccharum* has six species: *S. officinarum*, *S. spontaneum*, *S. robustum*, *S. sinense*, *S. barberi* e *S. edule*. Current sugarcane varieties are hybrids originating from interspecific crosses involving mainly 90% of *S. officinarum* and 10% of *S. spontaneum*. These hybrids are cited as *Saccharum* spp. (Ming et al. 2006).

America and Asia are the main sugarcane producing regions, such that in 2017 accounted for 55.7% and 37.2% of world sugarcane production, respectively (FAOSTAT

2019). The largest sugarcane producing country is Brazil, producing 758 Mt in 2017, about 41% of the world production. India, China, Thailand, Pakistan and Mexico are also important producers, contributing with 306, 104, 103, 73 e 57 Mt of sugarcane, respectively (FAOSTAT 2019).

The economic importance of this culture is related to its various purposes. Sugarcane is a raw material in the production of ethanol, biofuel widely used mainly in Brazil, in addition to the production of sugar and cane molasses, products for the food and feed industry; the vast market of products keeps its production growing continuously (Silalertruksa and Gheewala 2019).

Sugarcane is able to associate with a great diversity of diazotrophic plant growth-promoting bacteria, including species of the genera *Azospirillum* (Reis Junior et al. 2000; Tejera et al. 2005), *Azotobacter* (Tejera et al. 2005), *Burkholderia* (Perin et al. 2006; Antonio et al. 2016; Silva et al. 2016; Leite et al. 2018a, b), *Herbaspirillum* (Baldani et al. 1996; Reis Junior et al. 2000), *Pantoeae* (Taulé et al. 2012; Fischer et al. 2012, Silva et al. 2016), and the species *Gluconacetobacter diazotrophicus* (basonym *Acetobacter diazotrophicus*) (Cavalcante and Döbereiner 1988; Munöz-Rojas and Caballero-Mellado 2003; Restrepo et al. 2017), among others.

After the isolation and description of sugarcane-associated diazotrophic bacteria, and in view of the observed benefits of bacterial/plant association for other cultures, research has been intensified in Brazil. Dos Santos et al. (2018) observed the effects of inoculating a mix of diazotrophic bacteria (*G. diazotrophicus* PAL5T, *Herbaspirillum rubrisubalbicans* HCC10, *Herbaspirillum seropedicae* HRC54, *Nitrospirillum amazonense* CBAmC and *Paraburkholderia tropica* PPe4T) on sugarcane growth. After 15 days of planting, a 50% increase in dry mass of inoculated roots was observed.

The same group of bacteria was used in hydroponic sugarcane cultivation for 59 days under different concentrations of N. Two varieties of sugarcane were used: RB867515 (adapted to low fertility soils) and IACSP95-5000 (adapted to medium to high fertility soils). The authors reported that the two sugarcane varieties, when inoculated with the bacterial mix, presented different results regarding the activity of enzymes related to the assimilation of N. Under low N concentration, nitrate reductase activity was increased in RB867515 by 26% in the shoots, and by 48% in the roots, while glutamine synthetase activity was 21% higher than the control. For the IACSP95-5000 under low N concentration, nitrate reductase activity decreased by 62% in roots, and glutamine synthetase activity was increased by 16% (Dos Santos et al. 2019). This information corroborates with Schultz et al. (2017), who analyzed yield parameters in two field sites and with two sugarcane varieties (RB867515 and RB72454) inoculated or the same bacterial mix. For variety RB867515 the inoculation promoted increases in stem yield by 22.3 Mg ha^{-1} in the first site and 38.0 Mg ha^{-1} in the second site compared to the control. The variety RB72454 showed increases of 16.7 and 37.5 Mg ha^{-1}, respectively.

Optimum yield results via inoculation with the same bacterial mix suggest reduced N-fertilizer application. Pereira et al. (2018) consider that inoculation coupled with the application of a low dose of N (50 kg N ha^{-1}) can raise productivity with economy. In 2019 the first commercial inoculant for the sugarcane was released in Brazil, carrying *Nitrospirillum amazonense* strain.

Pastures with grasses and legumes

Estimates are that the global pasture area covers 26% of the ice-free land surface, but in many of these places, the pastures are degraded and insufficient to provide nutrients to the animals, demanding new areas (Steinfeld et al. 2006; Fonte et al. 2014). The major problem in increasing pasture areas is that they often occur in detriment of forests, leading to deforestation, decrease in biodiversity and other environmental damages (Steinfeld et al. 2006; Don et al. 2011).

In order to improve the development of grasses in degraded pastures, the use of PGPB is once again a viable strategy. The idea is to increase soil fertility, yield and nutritional quality of grasses, decreasing the pressures on native forests (Monk et al. 2009; Campos et al. 2012; Hungria et al. 2016).

Grasslands in Brazil are estimated in 180 million ha, of which over 60 million ha are classified as degraded (LAPIG 2018), with *Brachiaria* (=*Urochloa*) representing the main component (Hungria et al. 2016). Strains Ab-V5 and Ab-V6 of *A. brasilense* have been evaluated as inoculants for *Urochloa* spp. in different sites of Brazil and the combination with N-fertilizer (40 kg ha of N) increased biomass production by 15% and of protein by 25% in comparison to the control receiving only N-fertilizer (Hungria et al. 2016). Other studies confirmed the good performance of these strains of *A. brasilense* with brachiarias (Bulegon et al. 2016; Guimarães et al. 2016; Leite et al. 2018a, b), and also with another important pasture in Brazil, panicum *Panicum maximum*,=*Megathyrsus maximus*) (Leite et al. 2019). In addition to *A. brasilense*, positive results were reported for brachiaria inoculated with *Bacillus* sp. isolated from the rhizosphere of *Urochloa brizantha* (Araujo et al. 2012).

In New Zealand, Monk et al. (2009) isolated bacteria capable of colonizing the roots of tall fescue (*Festuca arundinacea*) grasses with promising characteristics for pastures. The isolated bacteria were studied in vitro and selected for their plant-growth promotion properties, such as the production of auxins, siderophores and P solubilization.

In Colombia, *Pennisetum clandestinum* (kikuyo) was inoculated with two PGPB strains of *Stenotrophomonas* sp. and *Pseudomonas* sp. able to synthesize indole compounds, to fix nitrogen and to solubilize phosphate in vitro. Under greenhouse conditions, significant increases in the biomass and root dry weight were observed in comparison to the non-inoculated control.

Pastures with legumes are also spread all over the world, and *Trifolium* spp., *Arachis pintoi*, *Medicago sativa* L., *Stylosanthes* spp. are important examples. Dozens of studies have been performed with PGPB with those legumes. *Trifolium repens* and *Trifolium pratense* are two clovers species broadly used in pastures in Uruguay. To ensure good development there is a recommendation, since 1967, of inoculation of both clovers with *R. leguminosarum* sv. *trifolii* strain U204, a commercial inoculant strain introduced from the USA (Tartaglia et al. 2019).

Alfalfa (*Medicago sativa* L.) is present in pastures in temperate and subtropical, and arid and semi-arid areas. Buntić et al. (2019) developed a liquid-formulated inoculant containing *Sinorhizobium* (=*Ensifer*) *meliloti* strain L3Si allowing better shelf life, pre-inoculation and performance in alfalfa, as there were no liquid inoculants available with this strain. Shoot N content of plants originated from seeds pre-inoculated 1 month before sowing ranged from 3.72 to 4.19%, whereas the control with N-fertilizer had 4.03%; the highest SDW value was of 27.12 mg/plant in the inoculated plants, higher than the control with N-fertilizer (20.20 mg/plant), indicating a high effectiveness of the liquid formulation (Buntić et al. 2019).

Interest in increasing alfalfa production has also growing in Saudi Arabia. Daur et al. (2018) isolated, identified and exploited the PGPR potential of 17 bacterial isolates belonging to the genus *Bacillus*, *Acinetobacter* and *Enterobacter* from the Saudi Arabia desert and evaluated their effects on alfalfa yield. The strains were single inoculated in alfalfa seeds and sown in the fields under desert conditions. All strains improved plant relative water content, chlorophyll (a and b), carotenoids, N, P and K contents, plant height, leaf-to-stem ratio and fresh and dry weight in comparison to the non-inoculated control. However, one major consideration in this and in several other studies is the need of regulation to avoid potentially pathogenic strains in microbial inoculants, such as *Acinetobacter*, *Enterobacter* and even some species of *Bacillus*.

In Brazil, forage peanuts (*A. pintoi*) and *Stylosanthes* spp. are the most commonly used legumes in pastures. For *A. pintoi*, two *Bradyrhizobium* spp. strains are used in commercial inoculants, SEMIA 6439 (=MGAP 13) and SEMIA 6440 (=NC 230). In a field experiment that resulted in the selection of these two strains, they increased shoot dry weight, in comparison to the non-inoculated controls, without and with N-fertilizer, by 63 and 47%, respectively (Purcino et al. 2003). More recently, estimates of BNF in *A. pintoi* under field conditions were up to 65% of the total N in plants in the spring period (Carvalho et al. 2019).

Despite the widespread use of *Stylosanthes* spp. in Brazil, there are still few studies about the diversity and symbiotic efficiency of nitrogen-fixing bacteria associated to this plant. Two strains have been used in commercial inoculants, *B. japonicum* SEMIA 6155 (=BR 502) and SEMIA 6154 (=BR 446); recently, SEMIA 6154 was recognized as the type strain of a new species, *Bradyrhizobium stylosanthis* (Delamuta et al. 2016). da Chaves et al. (2016) reported that two bacterial species isolated from *Stylosanthes* (strains ERR 1178 and ERR 942 of *Bradyrhizobium* spp.) in savanna areas in Roraima, Brazil, increased the shoot biomass and N of *Stylosanthes capitata* cv. Lavradeiro under greenhouse conditions.

Australia has a long-time tradition in selecting strains and inoculating forage legumes, with emphasis on *Trifolium* spp. (Brockwell et al. 1982; Collins et al. 2002; Yates et al. 2005). More recently, in the inland areas of central Queensland, *Leucaena* has been sown and provided excellent results as forage in animal production (Buck et al. 2019); however, the inoculation of this legume is still little studied in the country.

Vegetables

Vegetables can highly benefit from several PGPB, but this market niche is still not well explored. Taken as an example, tomato (*Solanum lycopersicum* L.) takes part in the diet of million people, consumed in salads, as ingredient of hot dishes and with great application in the industry as raw material in the manufacture of many products, mainly sauces (Subramanian 2016). Due to its versatility, tomatoes are one of the most produced vegetables worldwide. China accounts for one-quarter of world's tomato production, followed by India and the USA (Heuvelink 2018).

Tomatoes may respond to inoculation with *Azospirillum* (Alfonso et al. 2005; Mangmang et al. 2015a; Lima et al. 2018). In Colombia, inoculation with *A. brasilense* resulted in better seedling growth, plant nutritional status, and yield 11% higher than the non-inoculated control (Alfonso et al. 2005) (Table 1).

In India, PGPB of the genera *Bacillus* and *Azotobacter* were isolated from the rhizosphere of tomatoes and tested as inoculants for this crop (Prashar et al. 2014). Previous reports from Cuba show that inoculation of tomatoes seeds with *Azotobacter chroococcum* increased plant dry weight (Puertas and Gonzales 1999). In Brazil, positive effects of inoculation of two tomatoes cultivars with *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 have also been reported (Szilagyi-Zecchin et al. 2015), increasing shoot growth, chlorophyll a, b and total, and favoring the synthesis of indole compounds and siderophores.

Several other vegetables have been reported as responsive to microbial inoculants, including lettuce (*Lactuca sativa*) (Flores-Félix et al. 2013; Mangmang et al. 2014; Fasciglione et al. 2015), carrot (*Daucus carota* L.) (Flores-Félix et al. 2013; Clemente et al. 2016) and cucumber (*Cucumis sativus* L.) (Mangmang et al. 2015b). The increasing demands of the population on organic products may also stimulate the use of microbial inoculants for the production of vegetables.

Some of the actual threats for the use of microbial inoculants

Attention should be paid to some threats that appear from the increased scientific and commercial interest on microbial inoculants. Several studies are reporting plant-growth promoting benefits in studies with bacteria that may be harmful to plants, animals and humans. Analyzing these studies, there is no doubt that several strains of *Enterobacter* spp., of the *Burkholderia cepacia* complex, *Pseudomonas aeruginosa*, among others, can be isolated from soils and have the capacity of promoting plant growth (e.g. Adesemoye et al. 2008; Daur et al. 2018; Jung et al. 2018; Rojas-Rojas et al. 2019; Roychowdhury et al. 2019). However, they cannot be used as inoculants. Therefore, before proceeding with studies to verify the plant performance with such isolates, priority should be given to determine their taxonomic position.

In relation to agronomic practices, the compatibility with agrochemicals used for seeds treatments, with an emphasis on pesticides represents a major limitation to the survival of bacteria (e.g. Campo et al. 2009), and the problem has increased with the use of pre-inoculated seeds stored for long periods in contact with pesticides (Hungria and Mendes 2015). Priority should be given to the search for compatible agrochemicals and cell protectors (Hungria et al. 2005), or alternative technologies of application, such as the application of inoculants in-furrow to avoid the direct contact with the products used for seed treatment (Campo et al. 2010).

Amazingly, information about the benefits of microorganisms on plant growth is leading some farmers to the production of their own microbial inoculants and products for biological control. It is not difficult to perceive the threat that such practice can result to the agriculture. Production of microbial inoculants require specific requirements not easily followed even under specialized conditions (Hungria et al. 2005). Therefore, plant, human and animal pathogens have been found as predominant microorganisms in farmers´ products (Valicente et al. 2018; Hungria and Nogueira 2019) and may jeopardize the benefits of high-quality products.

Perspectives for the future

Research on inoculants and inoculation with rhizobia and legumes raised great interest from researchers and companies in the 1970s. In the following decades, although several reports of benefits of new PGPB and the advances achieved at the inoculant industry, modest interest from research and industry has been observed. However, nowadays, increased demand for food, interest in sustainable agriculture and increasing reports on pests and pathogens resistance to agrochemicals are exponentially raising the global interest on microbial inoculants. Based on the information presented in this brief review, it is possible to perceive the increased number of studies that have been carried out about the development of new inoculants (Santos et al. 2017; Gundi et al. 2018), identification of new strains, and new inoculation methods, e.g. Zvinavashe el al. (2019), who developed a protein-based biomaterial capable of encapsulating and protecting rhizobacteria inoculated into seeds even after sowing, improving the effects of inoculation. According to information from the Web of Science database, between 2015 and 2019, 68 papers (excluding revisions) were published using the keywords "inoculant" or "biofertilizer" followed by "production" or "development". Therefore, it is expected that in the following years innovation will be presented, encompassing both microorganisms and technologies. China currently leads the number of registered patents related to inoculation, more than 800, and India already has more than 100 inoculant industries (Fig. 2). It is expected that these numbers will also increase in other countries.

One challenge to the development of new inoculants relies on the increasing concerns about climate changes. The expected increases in temperature and dry periods in the next years will have major impacts on agriculture. According to Ramirez-Villegas and Thornton (2015), in tropical areas, maize and rice yields may decrease by 5–10% and 2–5%, respectively, for each degree of temperature increase. Climate changes will decrease the available areas for cultivation. It is therefore mandatory to search for microbial inoculants more effective under stressful conditions; on the other hand, microbial inoculants can also help to mitigate the effects of climate changes and other related abiotic stresses, such as salinity (e.g. Cerezini et al. 2016; Fukami et al. 2018b; Leite et al. 2018a, b). With increased availability of high-quality products, in addition to commitments from the governments towards more sustainable agricultural systems, the use of microbial inoculants is expected to dramatically increase in the following years.

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Abbreviations: All abbreviations have been cited in their complete forms when mentioned for the first time in the manuscript.

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Authors declare no ethical problems. The study has not involved any human or animal participation or data.

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CAPÍTULO 2

IMPACTO EXCEPCIONAL DAS ESTIRPES DE *Azospirillum brasilense* Ab-V5 e Ab-V6 NA AGRICULTURA BRASILEIRA: LIÇÕES DE QUE OS AGRICULTORES ESTÃO RECEPTIVOS PARA ADOTAR NOVOS INOCULANTES MICROBIANOS

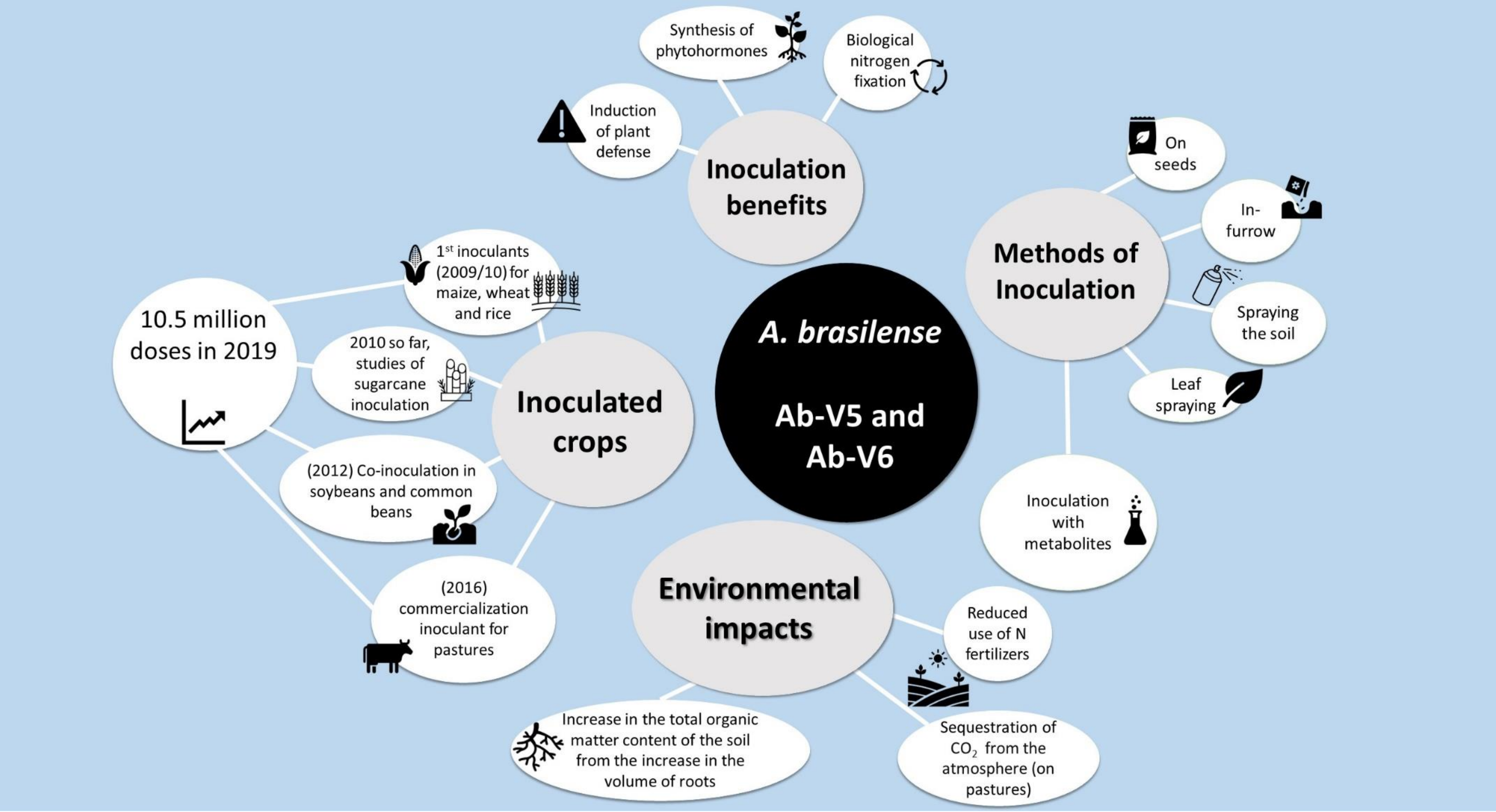
IMPACTO EXCEPCIONAL DAS ESTIRPES DE *Azospirillum brasilense* Ab-V5 e Ab-V6 NA AGRICULTURA BRASILEIRA: LIÇÕES DE QUE OS AGRICULTORES ESTÃO RECEPTIVOS PARA ADOTAR NOVOS INOCULANTES MICROBIANOS

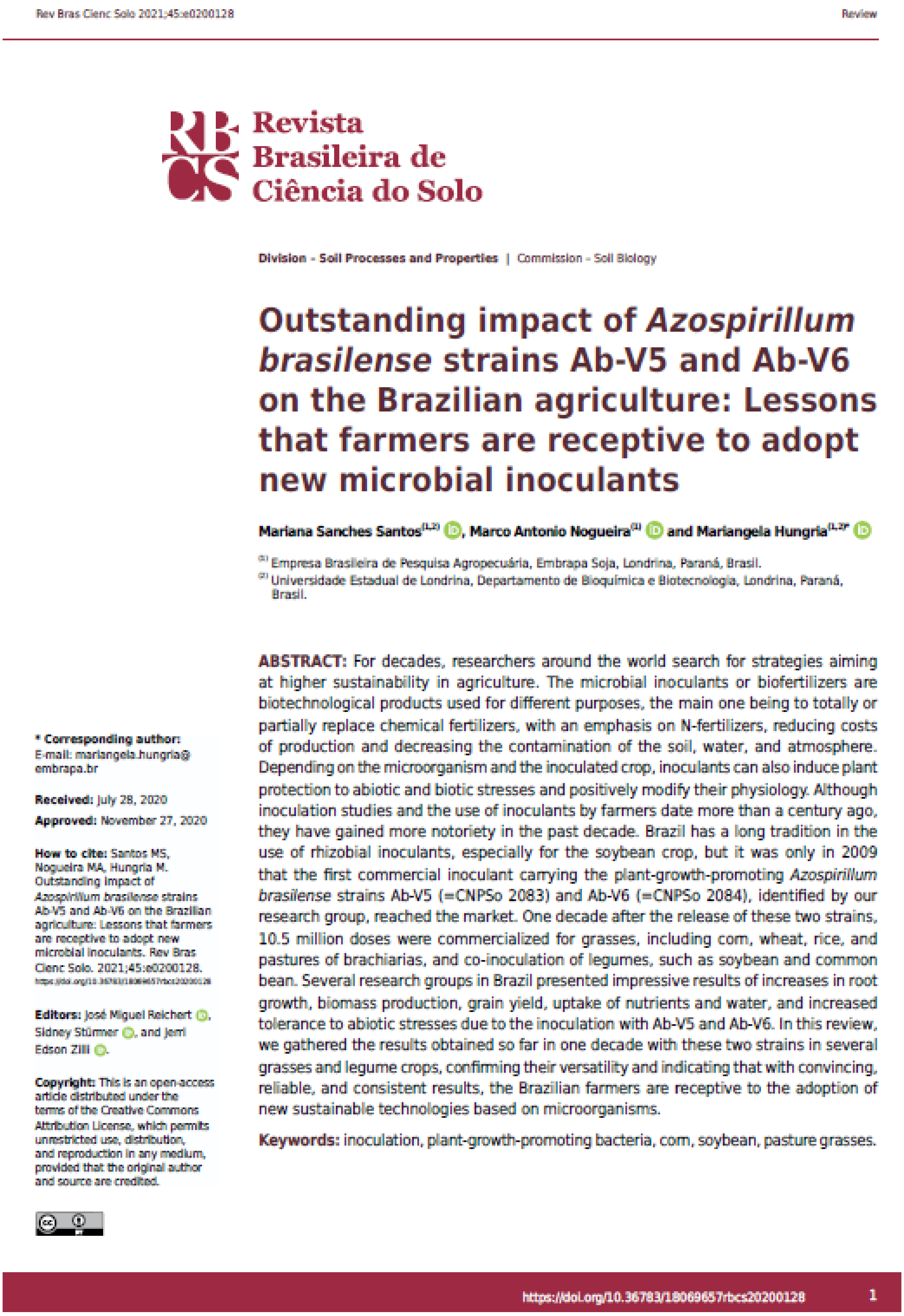
RESUMO

Durante décadas, pesquisadores de todo o mundo buscam estratégias visando uma maior sustentabilidade na agricultura. Os inoculantes microbianos ou biofertilizantes são configurados como produtos biotecnológicos com a principal função de substituir, total ou parcialmente, os fertilizantes químicos, com ênfase nos fertilizantes nitrogenados, reduzindo os custos de produção e diminuindo a contaminação do solo, da água e da atmosfera. Embora os estudos de inoculação e o uso de inoculantes pelos agricultores ocorram há mais de um século, na década passada ganharam mais notoriedade. O Brasil tem uma longa tradição no uso de inoculantes contendo rizóbios, especialmente para a cultura da soja, mas foi apenas em 2009 que o primeiro inoculante comercial com as estirpes Ab-V5 e Ab-V6 de *Azospirillum brasilense*, promotoras do crescimento de plantas identificadas pela pesquisa, chegou ao mercado. Uma década depois, foram comercializadas 10,5 milhões de doses para gramíneas, incluindo milho, trigo, arroz e pastagens de braquiárias e, também, para a coinoculação de leguminosas. Resultados impactantes de incrementos no crescimento radicular, na produção de biomassa e de grãos, na absorção de nutrientes e água, e aumento da tolerância a estresses abióticos devido à inoculação com Ab-V5 e Ab-V6 foram apresentados por vários grupos de pesquisa no Brasil. Nesta revisão, reunimos os resultados obtidos até o momento com essas duas estirpes em várias culturas leguminosas e não leguminosas, confirmando sua versatilidade e indicando que, com resultados convincentes, confiáveis e consistentes, os agricultores estão ansiosos por adotar tecnologias sustentáveis baseadas em microorganismos.

Palavras-chave: Inoculação, Bactérias promotoras do crescimento vegetal, Milho, Soja, Trigo, *Triticum aestivum*, *Urochloa*, *Zea mays*.

GRAPHIC ABSTRACT





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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

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ABSTRACT: For decades, researchers around the world search for strategies aiming at higher sustainability in agriculture. The microbial inoculants or biofertilizers are biotechnological products used for different purposes, the main one being to totally or partially replace chemical fertilizers, with an emphasis on N-fertilizers, reducing costs of production and decreasing the contamination of the soil, water, and atmosphere. Depending on the microorganism and the inoculated crop, inoculants can also induce plant protection to abiotic and biotic stresses and positively modify their physiology. Although inoculation studies and the use of inoculants by farmers date more than a century ago, they have gained more notoriety in the past decade. Brazil has a long tradition in the use of rhizobial inoculants, especially for the soybean crop, but it was only in 2009 that the first commercial inoculant carrying the plant-growth-promoting *Azospirillum brasilense* strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084), identified by our research group, reached the market. One decade after the release of these two strains, 10.5 million doses were commercialized for grasses, including corn, wheat, rice, and pastures of brachiarias, and co-inoculation of legumes, such as soybean and common bean. Several research groups in Brazil presented impressive results of increases in root growth, biomass production, grain yield, uptake of nutrients and water, and increased tolerance to abiotic stresses due to the inoculation with Ab-V5 and Ab-V6. In this review, we gathered the results obtained so far in one decade with these two strains in several grasses and legume crops, confirming their versatility and indicating that with convincing, reliable, and consistent results, the Brazilian farmers are receptive to the adoption of new sustainable technologies based on microorganisms.

Keywords: inoculation, plant-growth-promoting bacteria, corn, soybean, pasture grasses.

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INTRODUCTION

The biggest challenge for the agricultural sector is probably the capacity to produce food on a large scale to supply the increasing global demand. Limitations in finding new areas proper for cultivation and increasing reports of improper cultivation due to soil desertification and salinization led to the awareness of the importance of searching for sustainable agricultural systems and soil management, and new technologies are needed to reach these goals (Don et al., 2011; Campos et al., 2012; Fonte et al., 2014; Hungria et al., 2016).

Since the green revolution in the 1970s, the use of nitrogen (N) fertilizers is still a major practice used to increase food production, as by providing this nutrient, almost always higher yields are achieved (Pimentel, 1996; Khush, 1999). However, there are economic limitations to the use of N-fertilizers, related to (*i*) their high cost, as petroleum is used as the energy source for the synthesis of ammonia; (*ii*) the external dependence of many countries to their supply, e.g., Brazil imports more than 70 % of N-fertilizers; (*iii*) the low-efficiency use of N-fertilizers by plants, estimated at 30 to 50 %, depending on the crop, climate, soil, and management. Very important are also the environmental impacts of N-fertilizers, with great losses by volatilization, leaching, and denitrification, resulting in pollution of watercourses, ozone layer depletion, and global warming (Crispino et al., 2001; Moreira and Siqueira, 2006; Reis Junior et al., 2011; Reetz, 2017; Stewart and Lal, 2017; Hungria and Nogueira, 2019).

As a sustainable alternative to N-fertilizers, the use of microbial inoculants or biofertilizers increased, products containing live microorganisms named as diazotrophs, with the ability to establish different types of association with plants, providing N from the biological nitrogen fixation (BNF) process (Ormeño-Orrillo et al., 2013; De Bruijn, 2015; Kaschuk and Hungria, 2017). Rhizobia are symbiotic diazotrophic bacteria capable of forming close interactions with the host plant, resulting in the formation of specific structures, the nodules, in general in the roots, where the BNF process takes place (Evans and Burris, 1992; Moreira et al., 2010; Ormeño-Orrillo et al., 2013). Symbioses occur mainly with plants belonging to the family Fabaceae (= Leguminosae), e.g., between *Bradyrhizobium* spp. with soybeans (*Glycine max* (L.) Merr) and several species of *Rhizobium* with common bean (*Phaseolus vulgaris* L.) (Gomes et al., 2015; Hungria et al., 2015a). Currently, the great majority of the inoculants commercialized worldwide are for the soybean crop, with an emphasis on South America, especially Brazil and Argentina. In these South American countries, inoculants are applied every crop season and can fulfill soybean N needs, with no need of applying N-fertilizers (Hungria and Mendes, 2015; Hungria and Nogueira, 2019; Santos et al., 2019).

In addition to rhizobia, other non-symbiotic diazotrophic and also non-diazotrophic bacteria, usually classified as plant-growth-promoting bacteria (PGPB), may favor plant growth by a variety of processes, including the synthesis of phytohormones (Lin et al., 2012; Santi et al., 2013; Fukami et al., 2017, 2018a), phosphate solubilization (Rodriguez et al., 2004; Turan et al., 2012), biological control of pests and diseases (Correa et al., 2008), induction of plant tolerance to abiotic and biotic stresses (Yang et al., 2009; Bulgarelli et al., 2013; Cerezini et al., 2016; Fukami et al., 2018a,b), among others. Due to the broad range of benefits to plants, PGPB have also been increasingly used in agriculture worldwide (Santos et al., 2019).

One of the most well-known and studied PGPB is *Azospirillum*, and Brazil is a pioneer in studies with this genus. Initially named as *Spirillum* by Beijerinck (1925), *Azospirillum* had its nomenclature modified after the Brazilian researcher Johanna Döbereiner observed and described its ability to fix nitrogen when associated with grasses (Döbereiner, 1979). In 1978 the nomenclature "azo" was added as a prefix to the original name, in reference to the term "azote" used by Lavoisier for the element nitrogen. Besides, two species of the genus were described at that time, *A. lipoferum* and *A. brasilense* (Tarrand et al.,

1978) and were the subject of several studies in the following years. *A. brasilense* was first isolated in Brazil from the rhizosphere of *Digitaria decumbens* (Döbereiner and Day, 1976) and, since then, the country has maintained leadership in basic studies with *Azospirillum*, including taxonomy (Tarrand et al., 1978; Ferreira et al., 2020), ecology (Baldani and Döbereiner, 1980), quantification of the contribution of BNF (Döbereiner and Day, 1976; Döbereiner, 1979; Döbereiner and Pedrosa, 1987), and isolation of *Azospirillum* strains (Magalhães et al., 1983), but no commercial product was available in the country.

One main goal of our soil microbiology research group at Embrapa Soja is to select strains and develop microbial inoculants for application in agriculture. Initially, microbial inoculants and technologies were developed for the soybean crop (e.g., Hungria et al., 2006; Hungria and Mendes, 2015; Hungria and Nogueira, 2019). However, the success among farmers of the technologies launched with the soybean inoculant technologies developed, with an emphasis on the benefits of annual re-inoculation of the soybean crop, guaranteeing average grain yield increases of 8 % (Hungria and Mendes, 2015; Hungria and Nogueira, 2019; Hungria et al., 2020), resulted in the demand of microbial inoculants for other crops growing in rotation or succession with the soybean, especially corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.). In the late 1990s, our group started an evaluation of *Azospirillum* strains for these two cereals, that resulted in the identification of six strains able to promote grain yield increases (Hungria et al., 2010), and the first commercial inoculant was placed at the market in 2009. As Brazil has a long-time tradition of using two strains in commercial inoculants for the soybean crop (Hungria et al., 2006), the combination of *A. brasilense* strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084), elite natural variant strains obtained from *A. brasilense* strain Sp7, efficient for both cereals, started to be broadly evaluated and used in commercial inoculants, gaining notoriety and assuming an important role in the Brazilian inoculants market. Interestingly, the most used *Bradyrhizobium* strains in inoculants for the soybean crop in Brazil, SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7) are also natural variant strains adapted to the Brazilian soils and obtained in strain selection programs (Hungria et al., 1994; Hungria and Mendes, 2015). Research conducted in the last decade has shown the benefits of inoculation with Ab-V5 and Ab-V6 in other economically important grasses for Brazil including sugarcane (*Saccharum* spp.), rice (*Oryza sativa* L.), and pastures (Lopes et al., 2012, 2019; Hungria et al., 2016; Dos Santos et al., 2019; Heinrichs et al., 2020), in addition to the use in co-inoculation with rhizobia for legumes (Hungria et al., 2013, 2015b; Chibeba et al., 2015; Nogueira et al., 2018; Galindo et al., 2018, 2020a; Prando et al., 2019; Gericó et al., 2020; Rondina et al., 2020).

Considering the international market, the first commercial inoculant dates from 1910 in the USA, for the soybean crop. Nowadays, over one hundred years, soybean inoculants represent the great majority of the inoculants commercialized worldwide (Santos et al., 2019). In Brazil, estimates are that about 70 million doses of inoculants for the soybean crop were commercialized in the 2019/2020 crop season. Concerning the inoculants carrying *A. brasilense*, one of the first countries that released a commercial product was Argentina, in 1996, Nodumax-L, carrying *A. brasilense* strain Az39 (Cassán et al., 2020), followed by Mexico in 2002 (Reis, 2007). Currently, *A. brasilense* has been studied and used as inoculant in several countries, such as Uruguay, Egypt, India, and Colombia. In Brazil, an impressive increase in the number of commercialized doses has been seen in the short time of a decade, carrying strains Ab-V5 and Ab-V6 (Santos et al., 2019) (Figure 1).

Here we review the studies performed in this last decade in Brazil with strains Ab-V5 and Ab-V6 of *A. brasilense*, highlighting the market's receptivity for microbial inoculants replacing chemical fertilizers and, in specific cases, mitigating negative effects caused by abiotic and biotic stress. The increased use of inoculants carrying *A. brasilense* by the farmers confirms the profitability in grain yield of cereals, pastures, and legumes. Undoubtedly, the environmental benefits should also be considered.

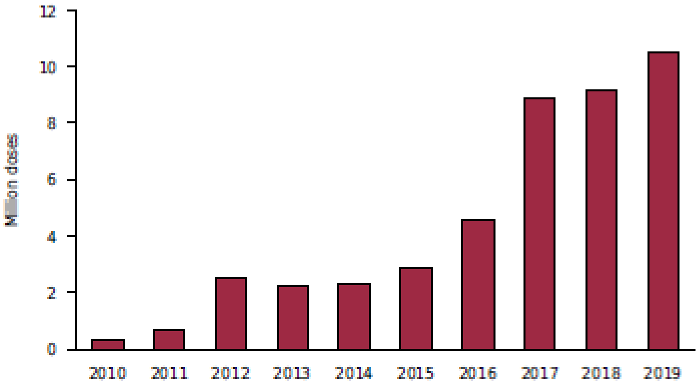

Figure 1. Doses of inoculants carrying *Azospirillum brasilense* strains Ab-V5 and Ab-V6 since the launch of the first commercial inoculant. Numbers based on data from Anpii (2020) and personal communication from the private sector.

Selection and validation of strains of *A. brasilense* for the corn and wheat crops

The benefits and economic gains generated from the soybean inoculation technology became better known and disseminated in Brazil in the mid-1990s (Hungria et al., 2006; Hungria and Mendes, 2015; Santos et al., 2019). Farmers then started to request studies for other non-legume crops, with an emphasis on corn and wheat, used in rotation or succession with the soybean. In 1996 our research group started in Paraná State studies to select strains for these two cereals. Strains were searched in the genus *Azospirillum*, broadly reported as PGPB with a variety of grasses (Döbereiner and Day, 1976; Döbereiner et al., 1976; Döbereiner, 1979; Döbereiner and Pedrosa, 1987; Paredes-Cardona et al., 1988). Currently, this is one of the most studied genera used as an inoculant because, in addition to BNF, *Azospirillum* spp. can contribute to plant development through other biological processes, including the synthesis of phytohormones (Tien et al., 1979; Fukami et al., 2017, 2018a), phosphate solubilization (Turan et al., 2012), and induction of tolerance to abiotic and biotic stresses (Bashan and De-Bashan, 2010; Cerezini et al., 2016; Fukami et al., 2018a,b; Santos et al., 2019).

The selection program of *Azospirillum* strains for commercial use in Brazil was carried out for eight years at Embrapa Soja, with evaluations under laboratory, greenhouse, and field conditions. In a first step, all *A. brasilense* and *A. lipoferum* strains available from several studies at the laboratory were evaluated for two properties: rates of acetylene reduction *in vitro*, in N-free semi-solid medium (laboratory), and capacity of promoting plant growth (greenhouse). Following, 17 field experiments were performed in Londrina and Ponta Grossa, Paraná State, southern Brazil, with the most promising strains. The first set comprised nine experiments with seed inoculation of single strains with peat inoculant, five with corn and four with wheat, resulting in the identification of six elite strains. Strains Ab-V4, Ab-V5, Ab-V6, and Ab-V7 showed increases in corn grain yield of 24 to 30 % compared to the non-inoculated control. For the wheat crop, the best strains were Ab-V1, Ab-V5, Ab-V6, and Ab-V8, increasing the yield by 13 to 18 % (Hungria et al., 2010).

Brazilian farmers have more than 70 years of tradition of using two strains in inoculants (Hungria et al., 1994; 2006). Therefore, in continuity to the studies, *A. brasilense* strains Ab-V5 and Ab-V6, efficient for both corn and wheat, were selected for a second set of eight field experiments, consisting of inoculating corn and wheat seeds with peat or liquid inoculant containing a combination of the two strains. In eight field trials in which N-fertilizer was applied at a low rate only at sowing, strains Ab-V5 and Ab-V6 contributed to average increases in yield of 27 % for corn and 31 % for wheat, when compared to the non-inoculated control (Hungria et al., 2010). The increase in productivity with strains Ab-V5 and Ab-V6 was suggested to be related mainly to the synthesis of phytohormones, resulting in root growth and increasing water and nutrients' absorption. However, precise evaluations were not performed at that time, and the suggestion came from the indications that N-uptake from soil was increased, while the contents of some soil nutrients (Ca, Mg, and N) in soil decreased (Hungria et al., 2010), in addition to visual observations of impressive root growth. The assumption was also supported by literature, rich in studies showing that *Azospirillum* improves root growth (Akbari et al., 2007; Vogel et al., 2013; Okon et al., 2015; Rondina et al., 2020; Santos et al., 2020a), impacting the uptake of water and nutrients.

The results with *A. brasilense* strains Ab-V5 and Ab-V6 were first presented in 2004 (Hungria, 2004), and confirmed in 2006 (Hungria et al., 2007), at the meeting of RELARE (Meeting of the Network of Laboratories for Recommendation, Standardization and Diffusion of Technology of Microbial Inoculants of Agricultural Interest), a committee joining research, industry and government to discuss issues related to microbial inoculants. The field trials obeyed all criteria defined at that time to be accepted as indicative of new strains for the production of inoculants in Brazil (Campo and Hungria, 2007), later incorporated in the Brazilian legislation for inoculants established by Mapa (Ministry of Agriculture, Livestock and Supply) (MAPA, 2010, 2011). In 2008 the strains were offered to the inoculant industries, and in 2009 the first inoculant produced in Brazil was released at the market by Stoller do Brasil SA named Masterfix L Gramineas® (Cassán et al., 2020); the company confirmed agronomic efficiency for the corn and rice crops. In the following year, an inoculant for the corn and wheat crops produced in public-private cooperation between Embrapa Soja and Total Biotecnologia, AzoTotal®, was released. Since then, Ab-V5 and Ab-V6 have been increasingly evaluated in experiments with several crops, including rice, sugarcane, pastures, and for co-inoculation of legumes. In 2016 there were 11 inoculants registered in Brazil with the combination of strains Ab-V5 and Ab-V6, the majority in liquid formulations (Cassán and Diaz-Zorita, 2016a). Considering the estimates that in 2010 about 300,000 doses were commercialized, and in 2012, 2.5 million doses, the increasing adoption by the farmers to about 10.5 million doses carrying these two strains in 2019/2020 is impressive. The number of commercial inoculants carrying *A. brasilense* also increases every year in Brazil, and efforts have been made to publish a list of registered products (Bioinsumos, 2020). This indicates that the farmers are anxious to use new technologies with economic benefits, resulting in grain yield increases and often in a reduction of chemical fertilizers.

Beneficial properties of Ab-V5 and Ab-V6

The excellent results obtained with the inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 resulted in a series of new studies investigating the main mechanisms that could explain their good performance.

In addition to describing for the first time the ability of *Azospirillum* to fix atmospheric N_2, Döbereiner (1979) also discussed conditions favoring the biological process, and the two main factors controlling BNF were identified as oxygen (O_2) and mineral N. According to the author, when the O_2 supply to the bacteria exceeds its consumption, assimilation of mineral sources of N are maximized and BNF is inhibited. In contrast, when the consumption of O_2 corresponds exactly to the amount needed, the conditions are optimum for nitrogenase synthesis and activity by the bacteria, and atmospheric N_2 is used as N source, if mineral N sources are not at inhibitory levels. In the absence of O_2, respiration is interrupted, ATP is not generated, and BNF does not occur. New information about strains Ab-V5 and Ab-V6 was obtained in 2018, with the sequencing of their genomes, estimated at 6,934,595 and 7,197,196 bp, respectively; with very similar genomes, both strains carry *nif* and *fix* genes, responsible for their ability to fix atmospheric nitrogen, and genes responsible for the synthesis of phytohormones (Hungria et al., 2018). Quantification of the contribution of BNF with strains Ab-V5 and Ab-V6 on BNF was poorly documented, when Araújo et al. (2015a) verified with the ^{15}N-isotope technique that inoculated corn with these two strains had BNF contributions of 19.4 % of total N accumulated in plants. Aguirre et al. (2020) reported that inoculation of *Cynodon dactylon* (L.) Pers. with these strains increased N in the grass by 7.4 %. However, despite the capacity of BNF, the major contribution of inoculation with *A. brasilense* Ab-V5 and Ab-V6 has been attributed to other plant-growth promotion effects (Hungria et al., 2010).

A. brasilense is capable of producing and secreting phytohormones in the rhizosphere, such as auxins, improving the development of the root system, stimulating its meristematic activity, and providing elongation and development of lateral roots (Ljung, 2013; Duca et al., 2014). With the development of the root system, the plant can absorb more water and nutrients, favoring its development and productivity (Tien et al., 1979; Akbari et al., 2007; Comas et al., 2012; Reece et al., 2015; Rondina et al., 2020). Fukami et al. (2018b), when analyzing secondary metabolites of Ab-V5 and Ab-V6 after 14 days of growth, verified the presence of indole-3-acetic acid (IAA), and acid salicylic (SA), while indole-3-lactic acid (ILA) and jasmonic acid (JA) were produced in relatively low amounts; the synthesis of gibberellic acid (GA_3) by Ab-V5 has also been reported by Fukami et al. (2017).

For legumes, the synthesis of phytohormones can contribute to greater nodulation (Hungria et al., 2013, 2015b; Chibeba et al., 2015; Rondina et al., 2020), because auxins can also stimulate the exudation of nodulation-inducing flavonoids, favoring the nodulation process (Star et al., 2012), and by increasing the volume of the roots, allowing greater contact surface with nodulating microorganisms (Rondina et al., 2020). The study developed by Rondina et al. (2020) shows these benefits; co-inoculation with *B. japonicum*, *B. diazoefficiens*, and *A. brasilense* (Ab-V5 and Ab-V6) resulted in significant changes in the morphology of the soybean roots, increasing the specific root length, root-hair length, and the number of root branches, in addition to the number of nodules, compared to the single inoculation with *Bradyrhizobium* spp.

Plants have several natural defense mechanisms induced when subjected to abiotic and biotic stresses (De Wit, 2007). One of the plant's main responses is the accumulation of reactive oxygen species (ROS) in plant tissues (Gill and Tuteja, 2010). Another example is the systemic acquired resistance (SAR), which confers resistance to plants against a broad spectrum of pathogens and is activated after infection (Fu and Dong, 2013). *Azospirillum* spp., as well as other PGPB genera, are capable of inducing different plant defense mechanisms, conferring resistance and helping against attacks by viruses, fungi, and pathogenic bacteria (Cassán et al., 2014). This mechanism is called "induced systemic resistance" (ISR) (van Loon and Bakker, 2005; Lugtenberg and Kamilova, 2009) and involves several physiological and biochemical changes in plants (Yang et al., 2009). Briefly, in the primary infected tissue, the bacterium triggers a plant reaction by emitting signals, pathogenesis-related proteins (PRs), which systematically spread in the whole plant, resulting in an increased defensive capacity, and the plant will remain protected for a long period (van Loon and Bakker, 2005; van Loon, 2007; Dutta et al., 2008). With the molecular tools available today is also feasible to consider genetic manipulation to insert this property in other bacteria.

It has been shown that strains Ab-V5 and Ab-V6 can induce defense mechanisms in the host plant (Fukami et al., 2018a). Under saline stress, inoculation resulted in increased production of salicylic acid (SA) in leaves and roots, and abscisic acid (ABA) in leaves. According to the authors, the increased synthesis of these compounds under stressful conditions provides plant protection since they are recognized as key signaling molecules regulating resistance in plants. In addition, it was also reported that, under normal cultivation conditions, Ab-V5 and Ab-V6 strains inoculated either at sowing or afterward by foliar spraying, as well as by foliar spraying of their metabolites, resulted in the induction of the activity of the enzymes superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), which are important agents against oxidative stress (Fukami et al., 2018b). *A. brasilense* strains Ab-V5 and Ab-V6, when inoculated in corn, can also interfere in the activation/repression of genes related to plant defense mechanisms such as the PRs reported for both strains (Fukami et al., 2017).

Another important feature in the metabolism of PGPB strains is the mechanism known as quorum sensing (QS). According to González and Marketon (2003), QS can be described as a communication mechanism between bacteria of the same or different species. This communication involves chemical mediators (autoinducers) and allows the cell to control certain genes' expression at high cell density. One of the most common auto-inducers is *N*-acyl-homoserine lactones (AHLs), synthesized by LuxI-type and detected by LuxR-type proteins, which activate the expression of target genes. The QS systems control important phenotypic responses, such as biofilm formation, bioluminescence, synthesis of exopolysaccharides (EPS), virulence factors, and antimicrobial compounds and motility, properties generally necessary to survive in the environment and to establish a relationship with eukaryotic hosts, both symbiotically and pathogenic (González and Keshavan, 2006; Pérez-Montaño et al., 2014).

Fukami et al. (2018c) described that strains Ab-V5 and Ab-V6 do not have a complete QS system, similar to other species of the same genus by Vial et al. (2006), as no *luxI* gene was found in their genomes. However, both Ab-V5 and Ab-V6 carry several *luxR* copies without any corresponding *luxI* that may recognize and respond to external AHL molecules (Fukami et al., 2018c). Upon contact with exogenous AHL molecules, the Ab-V5 QS mechanism affects biofilm formation, EPS synthesis, cell-swarming, and swimming phenotypes, in addition to benefits in solos growth. In contrast, Ab-V6 does not appear to use the QS mechanism, but the authors suggest that the larger production of IAA by the strain supplies this lack. Interestingly, although several differences between Ab-V5 and Ab-V6 have been reported, as almost all commercial inoculants carry both strains, differences in plant performance under field conditions that could be related to one or another strain have not been properly investigated yet.

Corn

Shortly after the publication of the study by Hungria et al. (2010), the interest in the inoculation of corn with strains Ab-V5 and Ab-V6 strains raised. In selecting the strains, the first objective was to reach small farmers that apply modest doses of N-fertilizers, farmers that cultivated short-cycle corn, and farmers growing wheat. Therefore, only a starter dose of N-fertilizer was applied at sowing, of 24 kg ha^{-1} of N for corn and 20 kg ha^{-1} of N for wheat, and resulted in not high, but compatible yields in the country, of 3,916 and 2,677 kg ha^{-1}, for the corn and wheat, respectively (Hungria et al., 2010), while the National averages in 2004 were of 3,291 and 2,227 kg ha^{-1}, respectively (Conab, 2020). However, farmers with higher technology questioned the ability of the strains to sustain higher corn yields. Following, experiments were performed with corn where, in addition to the application of N-fertilizer at sowing, a supplementation of 30 kg ha^{-1} of N (50 % of the recommended dose) was given, allowing to reach yields of up to 8,000 kg ha^{-1} (Hungria, 2011). Later, it was observed that yields higher than 8,000 kg ha^{-1} could be reached by inoculation, 24 kg ha^{-1} of N at sowing and 75 % of the recommended dose of N (67.5 kg ha^{-1} of N) topdressing at 30 days after emergence (Table 1).

Other Brazilian groups have also studied the effects of N-fertilization associated with inoculation with *A. brasilense* (Ab-V5 e Ab-V6) in corn in different regions of the country (Lana et al., 2012; Ferreira et al., 2013; Araújo et al., 2015b; Galindo et al., 2019a) (Table 1). An interesting study was carried out to understand whether inoculation of corn with *A. brasilense* Ab-V5 and different doses of N (low and regular) generated changes

Table 1. Studies reporting benefits of inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 in maize and wheat, and grain yield levels achieved in Brazil with different supplies of N-fertilizer

N at sowing	N topdressing	Grain yield	Site (State)	Reference
kg ha^{-1}				
			Corn	
24		3,905	Londrina and Ponta Grossa (PR)	Hungria et al. (2010)
24	30	>7,000	Londrina and Ponta Grossa (PR)	Hungria (2011)
-	90	6,785	Marechal Cândido Rondon (PR)	Lana et al. (2012)
-	90	7,604	Cascavel (PR)	
-	100	>8,000	Cerrado region (MG)	Ferreira et al. (2013)
30	-	9,531	Dourados (MG)	Araújo et al. (2015b)
30	90	9,862		
-	50	>8,500	Quirinópolis (GO)	Costa et al. (2015)
24	66	>8,000	Cachoeira Dourada (MG), Luis Eduardo Magalhães (BA) and Ponta Grossa (PR)	Fukami et al. (2016)
30	60	6,224	Maringá (PR)	Garcia et al. (2017)
-	100	>9,300	Cerrado region (MG)	Morais et al. (2016)
-	110	7,817	Campo Verde (MT)	Moreira et al. (2017)
32	100	>8,000	Selviria (MS)	Galindo et al. (2019a)
30	150	10,522	Selviria (MS)	Souza et al. (2019)
			Wheat	
20	-	2,656	Londrina e Ponta Grossa (PR)	Hungria et al. (2010)
24	36	5,610	Madre de Deus de Minas (MG)	Clemente et al. (2016)
24	36	3,997	Uberaba (MG)	
24	36	4,516	Lambari (MG)	
24	36	4,342	Patos de Minas (MG)	
24	67.5	>3,000	Ponta Grossa (PR)	Fukami et al. (2016)
-	150	3,544	Selviria (MS)	Galindo et al. (2017)
-	140	3,227	Santa Maria (RS)	Munareto et al. (2019)
-	100	>3,000	Selviria (MS)	Galindo et al. (2019b)
-	140	>3,000	Selviria (MS)	Galindo et al. (2020b)

in the diversity of its total and metabolically active endophytic bacterial community. For this, DNA and RNA analyses of the endophytic communities were performed and indicated that plants receiving low (30 kg ha^{-1} of N) or regular (160 kg ha^{-1} of N) doses of mineral N maintained similar diversity rates of the bacterial endophytes community. However, regarding the metabolically active community, the plants with the normal N level showed lower diversity than those of the low N level. Both treatments achieved similar productivity, showing that corn can perform well with lower rates of N-fertilizer when inoculated with strain Ab-V5 (Matsumura et al., 2015).

The reduction in N-fertilizer application combined with an inoculation to reach high yields results in economic and environmental impacts. The Brazilian area cropping corn in 2019/2020 was estimated at 18.44 million ha (Conab, 2020), and by decreasing the use of N-fertilizer by 25 %, considering a price of U$ 1 per kg of N, would save about U$ 440 million per year.

Effects of plant genotypes in the performance with *A. brasilense* have been long discussed (Stancheva and Dinev, 1992; De Salomone and Döbereiner, 1996; De Salomone et al., 1996). There are reports of different inoculation responses with Ab-V5 (Koltun et al., 2018; Zeffa et al., 2019) and Ab-V6 (Pereira et al., 2015) according to the corn genotype in experiments carried out under greenhouse and field conditions. Pereira et al. (2015) observed differences in N leaf content and root and shoot dry weight in different corn genotypes inoculated with Ab-V5 and Ab-V6. The same was observed by Marini et al. (2015) and Morais et al. (2016) for grain yield. In comparing root exudates released by corn hybrids with different responses to the inoculation with strains Ab-V5 and Ab-V6, Pereira et al. (2020) observed that the metabolites released by the less responsive hybrid reduced the amount of metabolites that served as bacterial energy, affecting bacterial metabolism in general. However, as Pereg et al. (2015) demonstrated, *Azospirillum* seems to interact and bring benefits for a large number of plant species. Therefore, although specific responses to the corn inoculation with Ab-V5 and Ab-V6 have been reported in different genotypes, the results obtained so far indicate that inoculation can be recommended to all genotypes.

Wheat

As with corn, research has been carried out with wheat after the selection and validation of strains Ab-V5 and Ab-V6. The studies followed similar objectives, including the choice of the most appropriate dose of N and time of application, alternative methods of inoculation, and differences between cultivars. As in other crops, in wheat N deficiency limits plant growth and grain yield. To attend the demands of the genotypes used in Brazil, with yields much lower than in temperate climates, on average of 2,723 kg ha^{-1} in 2019/2020 (Conab, 2020), farmers usually apply 60 to 120 kg ha^{-1} of N, depending on the N source, cultivar, soil properties, and climatic conditions; the fertilizer is split into two applications, at sowing and topdressing, approximately 30 days after emergence (Bona et al., 2016).

As with all grasses, despite the ability of *A. brasilense* to fix N_2, the amount is not sufficient to attend to the wheat´s needs, requiring supplementation of N-fertilizer. Studies performed with strains Ab-V5 and Ab-V6 in Brazil have shown compatibility with the N-fertilizer, since the application of 60, 100, 140, and 150 kg ha^{-1} of N resulted in gains in grain production (Clemente et al., 2016; Fukami et al., 2016; Galindo et al., 2017; 2019b; Munareto et al., 2019) (Table 1). Recently, Galindo et al. (2020b) described that inoculation, regardless of the N dose, guarantees higher accumulation of Mg and S in the straw, and of P, Ca, and Mg in the grains. Following, the same authors (Galindo et al., 2019c) reported that inoculation of wheat receiving silicon (Si) improves the uptake of N, highlighting another interesting strategy for the success of the inoculation.

In Brazil, it has been described that different wheat cultivars may have variable responses to inoculation with *A. brasilense* strain Ab-V5 alone (Lemos et al., 2013) and together with Ab-V6 (Feldmann et al., 2018), but this requires further investigation. Despite the positive results obtained so far with strains Ab-V5 and Ab-V6 in Brazil, in addition to other strains such as Sp7, Sp245, Sp246, Sp 262, Sp S82, Cd, M15, M16, M18, and M22 (Boddey et al., 1986, Baldani et al., 1986, 1987; Ferreira et al., 1987), evaluations with wheat are far behind those with corn, probably because of the small area and the low economic return of the crop in Brazil. However, the results reported in other countries, and a major example is Argentina (Cassán et al., 2015; Cassán and Diaz-Zorita, 2016b; Cassán et al., 2020), but also in Iran (Arzanesh et al., 2011), Russia (Shelud'ko et al., 2010), and Australia (Kazi et al., 2016) encourage the use of *A. brasilense* to increase the profitability and sustainability of the crop.

Rice

Rice, corn, and wheat are responsible for using approximately 50 % of the N-fertilizers consumed worldwide (Ladha et al., 2016). In Brazil, the strains Ab-V5 and Ab-V6 have also been used for the rice crop, although on a much lower scale than corn and wheat.

In the country, rice is cropped in two different systems, the rainfed cultivation, also called "upland cultivation", and the irrigated cultivation, occupying 367,000 and 1.298 million ha in the 2019/2000 crop season, respectively (Conab, 2020).

Garcia et al. (2016) evaluated the productivity of upland rice inoculated with *A. brasilense* strains Ab-V5 and Ab-V6. Four different doses, 0, 100, 200, and 300 mL ha^{-1} of an inoculant with the concentration of 2×10^8 cells mL^{-1}, and four methods of application (seeds, at sowing in-furrow, spraying of the soil immediately after sowing, and foliar spraying at the beginning of tillering) were evaluated. The best results were obtained when the plants were inoculated with 200 mL ha^{-1} of the inoculant, resulting in an increase of 10 % in yield in comparison to the non-inoculated control, with no differences between the methods of inoculation.

To evaluate the effects of flood irrigated rice inoculation, Dos Santos et al. (2019) inoculated seeds with Ab-V5 and Ab-V6 in addition to 81 kg ha^{-1} of N, split twice, corresponding to 60 % of the recommended dose for the crop. Grain yield of the treatment inoculated and receiving 60 % of the N-fertilizer was equal to that of plants receiving 100 % of the N dose, indicating the possibility of reducing in 40 % the application of N-fertilizer.

In another study comparing liquid and peat inoculants with Ab-V5 and AbV-6 in upland rice cultivation in four regions of Brazil, both types of inoculants were efficient, but the liquid inoculant showed the best results for yield. On average, plants inoculated with liquid inoculant had root dry mass of 32.4 g plant^{-1}, while on plants receiving peat inoculant, the average was 29.5 g plant^{-1}. The use of liquid inoculant in rice ensured average productivity close to 3,500 kg ha^{-1}, while for the peat inoculant the productivity was close to 3,000 kg ha^{-1} (Guimarães et al., 2020).

Sugarcane

Sugarcane was introduced in Brazil when the country was still a colony of Portugal, more than 500 years ago, as a strategy for the occupation of the territory and with the main objective of producing sugar. The crop adapted well to the Brazilian edaphoclimatic conditions, increasing the cropped area since then (Antunes et al., 2019). In 1975, sugarcane started to be also used as raw material for ethanol production as biofuel, towards decreasing the dependence on petroleum (Pazuch et al., 2017; Antunes et al., 2019; De Paula et al., 2019). Because the sugarcane biofuel is produced from renewable sources and is less polluting, its use is environmentally attractive. The expanded use of sugarcane increased the interest for the crop, such that today Brazil is the largest world producer, followed by India, China, and Thailand (FAO, 2018). In 2019/2020, the sugarcane production was estimated at 642.7 million tons, grown in an area of 8.44 million ha (Conab, 2020).

The doses of N-fertilized applied to the sugarcane in Brazil are modest, on average 45 kg ha^{-1} of N at planting and 80 kg ha^{-1} in the ratoon (new shoot at the base of sugarcane, after cropping). Interestingly, despite the low application, it has been observed that the accumulation of N by the culture is high, reaching up to 200 kg ha^{-1} in the sugarcane-plant cycle and 180 kg ha^{-1} in the ratoon (Urquiaga et al., 1992). This accumulation of N in quantities significantly higher than the doses applied stimulated the investigation of natural N replacement (Urquiaga et al., 1992, 2012). Several important Brazilian studies have been performed since the 1990s and suggested that BNF is greatly responsible for N supply to the plants, avoiding the depletion of N from the soil and ensuring productivity maintenance (Lima et al., 1987; Urquiaga et al., 1992, 2012; Oliveira et al., 2003, 2006). Urquiaga et al. (1992) provided convincing evidence that several sugarcane cultivars are capable of obtaining large and significant contributions of N from plant-associated diazotrophic bacteria. A variety of diazotrophic species started to be isolated, described, and studied, with an emphasis on *Acetobacter diazotrophicus* (Gillis et al., 1989; Reis et al., 1994; Kirchhof et al., 1998), later reclassified as *Gluconacetobacter diazotrophicus* (Yamada et al., 1997), and *Burkholderia tropica* (Reis et al., 2004), reclassified as *Paraburkholderia tropica* (Sawana et al., 2014). In Argentina, Tejera et al. (2005) reported that *Azospirilum* isolates showed specific associations and probably endophytic colonization of sugarcane.

In 2012, Lopes et al. (2012) evaluated 54 sugarcane families regarding inoculation with two inoculants carrying *A. brasilense*, the first with strains Ab-V5, Ab-V6, and Ab-V7 (named Triazo), and the other with *A. brasilense* strain IC26. There was no addition of N to any treatment or control. Different sugarcane families showed significantly different inoculation responses; however, in general inoculated plants performed better than the non-inoculated control. In a similar study conducted by Lopes et al. (2019), 27 sugarcane families were evaluated for inoculation with Triazo, and another inoculant containing a mix of bacteria isolated from sugarcane stems and roots, that included *G. diazotrophicus* Pal5, *Azospirillum amazonense* CBAmC (reclassified as *Nitrospirillum amazonense* by Lin et al., 2014), *Burkholderia tropica* Ppe8 (reclassified as *Paraburkholderia tropica*, Sawana et al., 2014), *Herbaspirillum rubrisubalbicans* HCc103, and *Herbaspirillum seropedicae* HRC54. The inoculation with Triazo showed better results than the mix of species for the plant length and diameter parameters. Gonçalves et al. (2020) investigated the interaction of sugarcane inoculation with strains Ab-V5, Ab-V6 and five doses of N-fertilizer (0, 30, 60, 90, and 120 mg dm^{-3}) in topdressing; the inoculation with *A. brasilense* benefited several growth parameters but, as expected, only when associated with N-fertilizer. Although there are still few studies with the inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 in sugarcane, the results are promising, especially by stimulating root growth, as shown in figure 2. In addition, there is a commercial inoculant at the Brazilian market carrying *N. amazonense* strain BR 11145.

Pasture grasses

In addition to agriculture, livestock is of great importance to the economy of Brazil. Beef production is estimated to reach 10.5 million tons in 2020 (USDA, 2020). In 2019, 8.7 million tons were consumed domestically, and 2.2 million tons were exported, mainly to China and Hong Kong. Most of the meat is exported *in natura*, followed by processed meats (Abiec, 2019). Livestock production in Brazil takes place mostly in pasture fields, which nowadays occupy about 180 million hectares (Mha), 60 Mha of them are natural grasslands (Unipasto, "Associação para o Fomento à Pesquisa de Melhoramento de Forrageiras Tropicais", unpublished data).

Figure 2. Sugarcane roots of variety RB 93 5744 grown under controlled greenhouse conditions in sandy soil and inoculated or not with *Azospirillum brasilense* strains Ab-V5 and Ab-V6, with different levels of mineral-N: (a) non-inoculated plants grown with 2 mg L^{-1} of N; (b) non-inoculated plants grown with 2 mg L^{-1} of N; (c) plants inoculated with Ab-V5 + Ab-V6 and receiving 2 mg L^{-1} of N. Photo: Dr. Leopoldo Sussumu Matsumoto.

A main and common limitation of livestock in pasture fields is soil degradation, resulting in a lack of nutrients, unable to meet the animal demands (Steinfeld et al., 2006; Fonte et al., 2014). In Brazil, estimates are that about 70 % of the pasture areas are at some level of degradation (Lapig, 2018; Embrapa, 2012). As deforestation should not be an option (Steinfeld et al., 2006; Don et al., 2011), increasing soil fertility, productivity, and nutritional quality of pasture grasses using PGPB may represent a key strategy (Campos et al., 2012; Hungria et al., 2016).

The majority of the areas under pastures in Brazil are with brachiarias (*Urochloa* spp. syn. *Brachiaria* spp.) that nowadays occupy about 86 Mha (Unipasto, unpublished data). The first commercial inoculant for pastures in Brazil was launched in 2016, carrying strains Ab-V5 and Ab-V6 as inoculants for *Urochloa brizanta* and *Urochloa ruziziensis*, also resulting from a public-private partnership of Embrapa Soja and Total Biotecnologia. The experiments to confirm the agronomic efficiency were performed in three different Brazil cities (Londrina-PR, Ponta-Grossa-PR, and Três Lagoas-MS) for three years, with 13 cuts per plant species. The authors highlighted the importance of comparing the performance of plants receiving N-fertilizer, as *A. brasilense* is not capable of supplying all plant N demand, and the main objective is to recover the fertility of soils with pastures. In comparison to the control receiving only N-fertilizer (40 kg ha^{-1} of N at sowing), when the N-fertilizer was combined with seed inoculation with *A. brasilense* strains Ab-V5 and Ab-V6, forage biomass production by *U. brizantha* and *U. ruziziensis* increased by 17.3 and 12.5 %, respectively (Hungria et al., 2016) (Table 2; Figure 3). Besides, N accumulation in shoots increased by an average of 25 % (Figure 3), indicating that the cattle would have not only more food but also a food of better quality. The increase of N in tissues was equivalent to a second application of 40 kg ha^{-1} of N-fertilizer. It is worth mentioning that a higher accumulation of dry matter implies an increase of CO_2 sequestered from the atmosphere estimated by the authors in 0.309 Mg ha^{-1} of CO_2-eq. This highlights another environmental benefit from inoculation since pastures are greatly responsible for the greenhouse gas (GHG) emissions in Brazil (Hungria et al., 2016).

In another study performed under field conditions in Araguaína, state of Tocantins, northern Brazil, Leite et al. (2019a) reported increases in the number of tillers, height, and dry mass of roots of *U. brizantha* inoculated with Ab-V5 and Ab-V6; the inoculant was at the concentration of 2 × 10^8 CFU mL^{-1} and seeds received 200 mL ha^{-1}. The authors

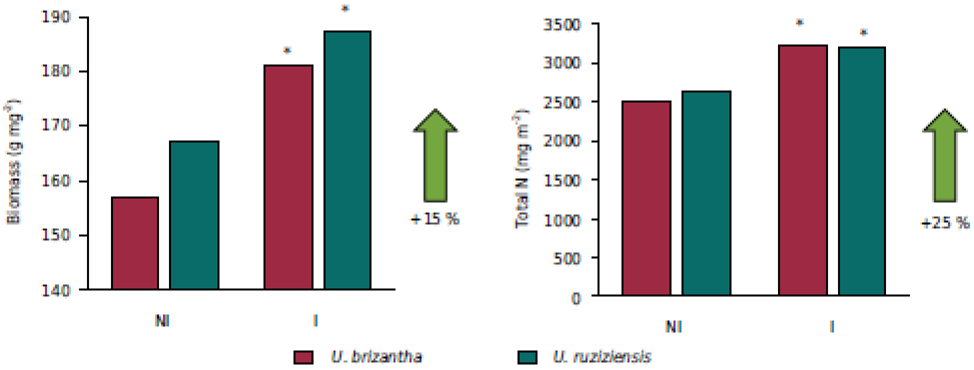

Figure 3. Shoot biomass production and total N accumulated in the biomass of *Urochloa* (=*Brachiaria*) *brizantha* and *Urochloa ruziziensis* inoculated or not with *Azospirillum brasilense* strains Ab-V5 and Ab-V6; all treatments received 40 kg of N ha^{-1} at sowing. Asterisks denote statistical difference between inoculated and non-inoculated ($p<0.05$, Tukey). Data represent the means of 13 cuts, each with four replicates, in three years for each pasture species. Modified from Hungria et al. (2016).

Table 2. Studies reporting benefits of inoculation with *Azospirillum brasilense* strains Ab-V5 and Ab-V6 in pastures in Brazil

Crop	Fertilization (N)	Trial	Result	Reference
Urochloa brizanta	40 kg ha^{-1}	Field	Biomass increased 17.3 % compared to the fertilization alone	Hungria et al. (2016)
	50 kg ha^{-1}	Field	Height increased by 4.16 %, biomass by 32.8 % and dry mass by 41.24 % compared to the fertilization alone	Rocha and Costa (2018)
	50 kg ha^{-1}	Field	Increase in the number of tillers, height, and root mass	Leite et al. (2019a)
	25 kg ha^{-1}	Field	Root dry mass increased 36 % compared to the non-inoculated	Heinrichs et al. (2020)
Urochloa ruziziensis	40 kg ha^{-1}	Field	Biomass increased 12.5 % compared to the fertilization alone	Hungria et al. (2016)
	20 kg ha^{-1}	Greenhouse	Improvement in the duration and rate of leaf renewal	Duarte et al. (2020a)
Cynodon dactylon	100 kg ha^{-1}	Field	Inoculated and fertilized pastures with 100 kg ha^{-1} N showed similar forage yield to non-inoculated pastures that received 200 kg ha^{-1} N	Aguirre et al. (2018)
Megathyrsus maximus	-	Field	Forage accumulation increased 36 %, leaf N 9 %, and root mass 96 %	Leite et al. (2019b)
	200 mg dm^{-3}	Greenhouse	Increase in dry weight of shoot and root, number of tillers, and relative chlorophyll index	Sá et al. (2019a)
	100 mg dm^{-3}	Greenhouse	Increase in dry weight yield of shoots and roots, compared to the non-inoculated control without N-fertilizer	Lima et al. (2020)
	50 kg ha^{-1}	Greenhouse	Increase in shoot dry mass and total N, P, Ca, and Mg accumulated in tissues	Picazevicz et al. (2020)

estimated that the inoculation with Ab-V5 and Ab-V6 allowed the reduction of 20 % in the need for N fertilizer.

Increases in *U. brizantha* root dry weight with the inoculation of Ab-V5 and Ab-V6 in addition to 25 kg ha^{-1} of N were also observed by Heinrichs et al. (2020), of up to 36 % when compared the non-inoculated and fertilized control. In another analysis, the authors demonstrated that, in the case of plants that did not receive N-fertilizer, the root dry weight values were 3,095 kg ha^{-1} for non-inoculated plants and 3,532 kg ha^{-1} for inoculated plants, statistically different. Rocha and Costa (2018) also observed that the inoculation of *U. brizantha* and 50 kg ha^{-1} of N contributed significantly to increases in height, chlorophyll content, biomass, dry mass, and number of tillers in comparison to plants receiving only N-fertilizer (Table 2).

As commented before, *A. brasilense* can improve plant tolerance to abiotic stresses, and good performance under water stress was reported in pastures inoculated with strains Ab-V5 and Ab-V6, including *U. ruziziensis* (Bulegon et al., 2016, 2019) and *U. brizantha* (Leite et al., 2019a). Moreira et al. (2020) reported the results obtained in an experiment performed under greenhouse conditions with *U. brizantha*, evaluating the inoculation with Ab-V5 and Ab-V6 at different doses of application, 5, 10, 20, and 40 mL kg^{-1} (2 × 10^8 CFU mL^{-1}) and time of watering. Some plants were watered two days after sowing, others after 4, 8, and 16 days. The best conditions for growth and development of *U. brizantha* were obtained in low doses, 5-10 mL kg^{-1}, and when the plants were watered until four days after sowing. It is worth mentioning that several PGBP, due to the synthesis of high amounts of phytohormones, especially IAA, should not be applied in higher doses, as instead of promoting, bacteria can inhibit plant growth.

This effect was also reported for Ab-V5 and Ab-V6 with common bean (Hungria et al., 2013) and soybean (Braccini et al., 2016).

Also, under greenhouse conditions, Sá et al. (2019b) reported increases in shoot and root dry weight, as well an in relative chlorophyll index and N uptake of *U. ruziziensis* inoculated with Ab-V5 and Ab-V6, while Duarte et al. (2020a), in pots filled with sandy soil, observed that the inoculation of *U. ruziziensis* with these two strains improved mainly the duration and rate of renewal of leaves.

The next step was to evaluate the application of strains Ab-V5 and Ab-V6 in pastures of *Urochloa* already established, and again, positive results were obtained, with average increases in biomass production of 20.9 % considering seven field trials performed in two sites in the state of Paraná, Brazil, in addition to increases in the contents of N and K in shoots (Hungria et al., unpublished data), and the technology was released in 2020.

In addition to *Urochloa* spp., the benefits of inoculation with Ab-V5 and Ab-V6 have also been described for other pastures, including panicum (*Megathyrsus maximus* syn. *Panicum maximum*) (Leite et al., 2019b; Sá et al., 2019a; Carvalho et al., 2020; Lima et al., 2020; Picazevicz et al., 2020) that nowadays occupies about 30 Mha (Unipasto, unpublished data) and the coast-cross grass (*Cynodon dactylon*) (Aguirre et al., 2018) (Table 2).

In conclusion, the inoculation of forage grasses with *A. brasilense* Ab-V5 and Ab-V6 probably represents the most promising technology for increasing the sustainability and productivity of millions of hectares with pastures in Brazil, contributing to increases in root and shoot biomass, N concentration in shoots, number of tillers, among others, and allowing the partial replacement of N-fertilizers (Duarte at al., 2020b). In addition, inoculation can play a very important role in combining animal production and environmental conservation efforts, as it improves plant nutrition, promotes soil conservation and fertility as well as for carbon sequestration.

Co-inoculation

The global inoculant market has been seeking new strains, the development of new formulations, and the validation of application methods. In the last decade, the idea of inoculants combining different species of microorganisms that contribute by different microbial processes has gained attention, in a practice that has been called as mixed inoculation or co-inoculation. The most studied combinations include symbiotic rhizobia together with PGPB showing other properties, such as *A. brasilense* strains efficient in synthesizing phytohormones. There are currently a variety of co-inoculants on the market for many crops (Santos et al., 2019).

In Brazil, strains Ab-V5 and Ab-V6 have been studied in co-inoculation with rhizobia for soybeans (Hungria et al., 2013, 2015b; Chibeba et al., 2015; Braccini et al., 2016; Ferri et al., 2017; Nogueira et al., 2018; Galindo et al., 2018; Prando et al., 2019; Rondina et al., 2020), common beans (Hungria et al., 2013), cowpea (*Vigna unguiculata* L. Walp.) (Galindo et al., 2020a, 2021), peanut (*Arachis hypogaea* L.) (Silva et al., 2017; Freitas et al., 2020; Gericó et al., 2020), and alfalfa (*Medicago sativa* L.) (Silva et al., 2020). Interestingly, rhizobia have also been studied in association with Ab-V5 and Ab-V6 in corn (Dartora et al., 2016; Fukami et al., 2018d), showing significant improvements in plant development when compared to the single inoculation with *A. brasilense*.

In soybean cultivation in Brazil, the co-inoculation with *Bradyrhizobium* spp. and *A. brasilense* proved to be more advantageous than single inoculation with *Bradyrhizobium* spp. In areas that have been inoculated before and showed an established population of soybean bradyrhizobia, Hungria et al. (2013) observed an average increase in soybean grain yield of 16.1 % by co-inoculation (*B. japonicum* strains SEMIA 5079 and

B. diazoefficiens SEMIA 5080 and *A. brasilense* strains Ab-V5 and Ab-V6), whereas the single inoculation with *Bradyrhizobium* spp. increased yield by 8.4 %, both compared to the non-inoculated control. Consequently, Ab-V5 and Ab-V6 guaranteed twice the increase in productivity provided by single inoculation with *Bradyrhizobium*. Again, the first commercial inoculant was released in a public-private partnership of Embrapa Soja and Total Biotecnologia in 2013. Similar results were observed by Galindo et al. (2018), in this case with soybean co-inoculated with *Bradyrhizobium elkanii* (SEMIA 5019), *B. japonicum* (SEMIA 5079), and *A. brasilense* (Ab-V5 and Ab-V6), with a yield increase of 11.2 % in comparison to the single inoculation with *Bradyrhizobium*. Ferri et al. (2017) reported that the co-inoculation of soybean with *B. japonicum* SEMIA 5079, *B. elkanii* SEMIA 5019 and Ab-V5 and Ab-V6 resulted in 20.3 % increase in grain yield in comparison to the single inoculation with *B. japonicum*. It is worth mentioning that foliar spray of *A. brasilense* Ab-V5 and Ab-V6 at the vegetative stage of soybean also improved nodule number and dry weight, plant height, and the number of pods and grains (Toniato et al., 2020).

Given the positive results obtained with the co-inoculation of soybean with Ab-V5 and Ab-V6, a large-scale program of transference of the technology to the farmers has begun in the state of Paraná, in a partnership between Embrapa Soja and Emater ("Empresa Paranaense de Assistência Técnica e Extensão Rural"). The program started in 2017 and consists of four stages: (*i*) training extension technicians; (*ii*) installation and monitoring of technical reference units; (*iii*) technical meetings for the dissemination of technology; (*iv*) collection, tabulation, and analysis of the results obtained (Nogueira et al., 2018; Prando et al., 2019). In 2017/2018, 37 reference units were established in 23 municipalities and attended 665 farmers. Single inoculation with *Bradyrhizobium* and co-inoculation with Ab-V5 and Ab-V6 resulted in yield gains of 1.8 and 5.6 bags of 50 kg ha^{-1}, respectively, with net profits in the Brazilian money "reais" of R$ 126.60 and R$ 390 ha^{-1}, respectively (Nogueira et al., 2018). Similar results were obtained in the following crop season, 2019/2020, with 61 reference units in 46 municipalities attending 925 farmers, with co-inoculation resulting in a net profit of R$ 296 ha^{-1} (Prando et al., 2019). Noteworthy, despite the very short time of launching the co-inoculation technology for the soybean crop, the adoption by the farmers impressive increases every year, for example, from 15 % in the 2018/2019 to 25 % in the 2019/2020 cropping season; the adoption takes place faster in the North region (Table 3).

Other relevant increases in grain yield were achieved by Galindo et al. (2020a) in cowpea co-inoculated with Ab-V5 and Ab-V6, with 25.22 % increase in yield, in comparison to single inoculation with *Bradyrhizobium* sp. Following, in another study with cowpea, when compared to the single inoculation with *Bradyrhizobium*, co-inoculation with strains Ab-V5 and Ab-V6 increased N use efficiency by 35.5 %, as well as N recovery and N accumulation, altogether leading to improved crop growth; furthermore, co-inoculation also provided a positive residual effect on wheat, increasing yield by 5.8 % (Galindo et al., 2021). Impacting increases were also reported with co-inoculation of common bean with *Rhizobium tropici* SEMIA 4080 (=PRF 81) and Ab-V5, Ab-V6, with an average increase in grain yield of 19.6 %, in comparison to 8.3 % by single inoculation with rhizobium (Hungria et al., 2013).

The improvement in the development of the root system by *A. brasilense* Ab-V5 and Ab-V6, as shown for the soybean (Rondina et al., 2020), is probably a major factor contributing to the increased uptake of water and nutrients, resulting in higher yields in relation to the single inoculation with rhizobia. In addition, benefiting water absorption (Silva et al., 2019; Freitas et al., 2020; Naoe et al., 2020) may increase tolerance to moderate periods of water stress (Cerezini et al., 2016; Freitas et al., 2020). Furthermore, by improvements in the root system, Ab-V5 and Ab-V6 may promote early nodulation, as shown for the soybean (Chibeba et al., 2015; Cerezini et al., 2016).

Table 3. Percentage of adoption of soybean single inoculation with *Bradyrhizobium* spp. and co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense* by the Brazilian farmers in the main producing states of Brazil. According to Anpii (Associação Nacional dos Produtores e Importadores de Inoculantes) and Spark smarter decisions

State	2018/2019		2019/2020	
	Inoculation	Co-Inoculation	Inoculation	Co-Inoculation
South Region				
Rio Grande do Sul	73	7	61	11
Santa Catarina	81	11	78	14
Paraná	71	12	68	11
Southeast Region				
São Paulo	86	7	83	21
Minas Gerais	86	15	80	14
Central-West Region				
Mato Grosso do Sul	83	17	84	23
Mato Grosso	85	16	85	36
Goiás-Federal District	86	19	80	25
North Region				
Rondônia	93	15	91	no information
Pará	100	30	100	80
Tocantins	92	34	100	77
Northeast Region				
Maranhão	95	26	100	40
Piauí	99	32	100	21
Bahia	97	8	93	14

Methods of inoculation

The use of pesticides and fungicides is a well-established practice in crop management. Pesticides are estimated to be applied in 85 % of the world's agricultural grain production to protect plants against pests and diseases (Kim et al., 2017). These products can be applied directly to the seeds or in the sowing furrow and later on leaves, by spraying. With the increased use of inoculants on seeds, the compatibility with agrochemicals has also been increasingly questioned. It is well known that bacterial cells of the inoculants can suffer from the toxicity of chemical compounds present in pesticides and other agrochemicals, often resulting in drastic cellular mortality, and impairing the effectiveness of the inoculant (Dunfield et al., 2000; Campo et al., 2009). The same incompatibility has been observed for strains Ab-V5 and Ab-V6, inoculated in corn seeds treated with pesticides (Santos et al., 2020a,b), which may impair the benefits of the PGPB. To minimize the toxic effects of pesticides, alternative methods of inoculation to avoid the direct contact between the microorganisms and the pesticides have been investigated. Some of the studied methods include the inoculation in-furrow, by spraying the soil at sowing, and by leaf spraying in seedlings. These three inoculation methods with Ab-V5 and Ab-V6 were studied in corn, and in the case of leaf spray applied at the V2.5 stage of the plant growth cycle (Fukami et al., 2016). Different doses of inoculant were evaluated, with one dose corresponding to the application of 1.0×10^5 cells seed^{-1}, and the plants were also N-fertilized at 100 or 75 % of the recommended dose. Preliminary tests were carried out in a greenhouse and subsequently in the field in different producing areas of Brazil. All three alternative methods of inoculation proved to contribute to improving yield, with the best results achieved with the application of 2 and 4 doses in-furrow or by leaf spray, with gains of up to 773 kg ha^{-1} even with the reduction to 75 % of the N-fertilizer (Fukami et al., 2016).

The same three alternative methods of inoculation were investigated in wheat, with the spray applied at the third tiller, and one dose corresponding to the application of 1.74 × 10^{4} cells seed^{-1} plant^{-1}. The best results with 75 % of N-fertilizer were obtained with the leaf spray of two doses, achieving yields higher than 3,000 kg ha^{-1} (Fukami et al., 2016). Positive results for wheat inoculation with Ab-V5 and Ab-V6 (concentration of 2 × 10^{8} cells mL^{-1}) via leaf spray were also observed by Correia et al. (2019), and in comparison to the treatment receiving 50 % of the N-fertilizer, leaf spray of 300 mL 100 kg^{-1} seeds increased yield by 184 kg ha^{-1}. Galindo et al. (2019b), when comparing the three methods of wheat inoculation, seeds, in-furrow at sowing and foliar consisting of 300 mL ha^{-1} (2 × 10^{8} CFU mL^{-1}) in a field experiment in the Cerrados, reported that although the highest grain yield (26.7 % over the non-inoculated control) was achieved with seed inoculation, good results were obtained with the two alternative methods.

Industrial development

Given the positive results reported by the inoculation of different crops with strains Ab-V5 and Ab-V5, a demand raised for new inoculant formulations, as the cell concentration achieved is lower than in rhizobial inoculants and the shelf-life is shorter. However, few studies have been developed in the country for this purpose (Marcelino et al., 2016; Oliveira et al., 2017; Santos et al., 2017; Vercelheze et al., 2019). Factors such as biofilm production, maintenance of the pH in the medium, encapsulation of bacterial cells, protection against external agents, and easy use can be evaluated and may result in improved formulations (Kumaresan and Reetha, 2011; Trujillo-Roldán et al., 2013; Bashan and de-Bashan, 2015; Marcelino et al., 2016; Santos et al., 2017). Besides that, there is a demand to improve the compatibility with agrochemicals and the possibility of pre-inoculation of seeds.

The addition of microbial metabolites to improve inoculant performance has also been investigated. For example, by applying metabolites of strains Ab-V5 and Ab-V6 via leaf spraying, Fukami et al. (2017) obtained significantly higher expression of genes related to stress tolerance and defense against pathogens, indicating that the use of their metabolites can be better explored.

Final remarks

Based on the information presented in this review, we may conclude that *A. brasilense* strains Ab-V5 and Ab-V6 have gained prominence in Brazilian agriculture in a very short time (Figures 1 and 4). The great versatility of both strains, contributing to a variety of biological processes, opens opportunities to extend the evaluations to several other plant species cropped in the country. This review shows that elite strains of plant-growth-promoting bacteria with good performance are easily accepted and adopted by the farmers. One advantage of Brazil is that several farmers are familiar with the concept of microbial inoculants, such that the efforts towards education about microbial bioproducts should now be directed to small farmers with less access to technical information. In addition, the success achieved in Brazil can stimulate studies and application in other countries with local strains.

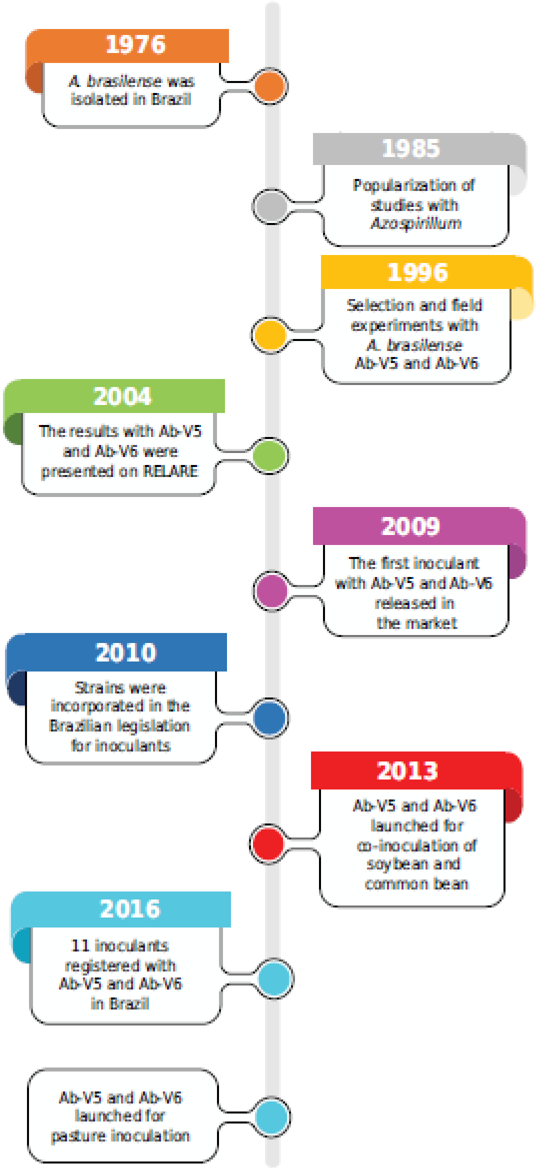

Figure 4. Chronology of some important steps in the prospection, identification, and release of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 in Brazilian agriculture.

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CAPÍTULO 3

MÉTODO PARA RECUPERAÇÃO E CONTAGEM DE CÉLULAS VIÁVEIS DE *Azospirillum brasilense* INOCULADAS EM SEMENTES DE MILHO

MÉTODO PARA RECUPERAÇÃO E CONTAGEM DE CÉLULAS VIÁVEIS DE *Azospirillum brasilense* INOCULADAS EM SEMENTES DE MILHO

RESUMO

A inoculação de sementes com bactérias fixadoras de nitrogênio e promotoras de crescimento de plantas é uma prática agrícola bem estabelecida, cada vez mais adotada em todo o mundo, diminuindo os custos e os impactos ambientais da produção de alimentos. A maioria dos inoculantes comercializados globalmente é para a cultura da soja, e um método para recuperação de células de *Bradyrhizobium* de sementes de soja inoculadas para contagem subsequente foi adotado por vários laboratórios da América do Sul, especialmente para investigar a sobrevivência bacteriana em sementes tratadas com pesticidas. Entretanto, o uso de inoculantes contendo *Azospirillum brasilense* em culturas de cereais aumentou exponencialmente, exigindo investigação sobre a recuperação e contagem de células de sementes inoculadas. Neste trabalho, verificamos que o método utilizado para recuperação e contagem de células viáveis de *Bradyrhizobium* em sementes de soja inoculadas com *Bradyrhizobium* não era aplicável a sementes de milho inoculadas com *A. brasilense*. Em seguida, modificamos várias etapas do método, com o objetivo de recuperar células viáveis de *Azospirillum*. A principal limitação foi identificada na natureza do tegumento das sementes de milho, seco e pobre em nutrientes, resultando na agregação celular de *A. brasilense*. A pré-hidratação das sementes por 2 h em água destilada estéril, seguida de agitação por 30 minutos em água destilada estéril com Tween 80, permitiu a contagem adequada de células de *A. brasilense* recuperadas das sementes de milho. O método foi aplicado com sucesso para contar células de *Azospirillum* recuperadas de sementes de milho pré-inoculadas e para estimar o impacto do tratamento de sementes com pesticidas na sobrevivência celular.

Palavras-chave: Inoculante, recuperação celular, *Zea mays*, *Azospirillum*, pesticidas.

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Method for Recovering and Counting Viable Cells from Maize Seeds Inoculated with *Azospirillum brasilense*

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Abstract

The inoculation of seeds with nitrogen-fixing and plant-growth promoting bacteria is a well-established agricultural practice that has been increasingly adopted worldwide, decreasing costs and environmental impacts of food production. Most of the globally commercialized inoculants are for the soybean crop, and a method for recovery of *Bradyrhizobium* cells from inoculated soybean seeds for subsequent counting has been adopted by several laboratories of South America, especially to investigate the bacterial survival on seeds treated with pesticides. However, the use of inoculants containing *Azospirillum brasilense* in cereal crops has exponentially increased, requiring investigation about the recovery and counting of cells from inoculated seeds. We first verified that the method used for recovery and counting of viable cells of *Bradyrhizobium* from soybean seeds was not applicable for maize seeds inoculated with *A. brasilense*. We then modified several steps of the method, aiming at succeeding in recovering *Azospirillum* viable cells. The main limitation was identified in the nature of the seed tegument, dry and poor in nutrients, resulting in *A. brasilense* cell aggregation. Pre-hydration of seeds for 2 h in sterile distilled water, followed by shaking for 30 min in sterile distilled water with Tween 80 allowed proper counting of *A. brasilense* cells recovered from maize seeds. The method was successfully applied to count *Azospirillum* cells recovered from pre-inoculated maize seeds, and to estimate the impact of seed treatment with pesticides on cell survival.

Keywords: Inoculant, cell recovery, *Zea mays*, *Azospirillum*, pesticides.

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INTRODUCTION

Concerns about environmental sustainability have resulted in an increasing use of microorganisms in agriculture, aiming at partially or fully replace agrochemicals, with emphasis on fertilizers. Inoculants, also called as biofertilizers in some countries, are products containing living microorganisms that, when applied to the seeds, plant surface, or soil, are able to colonize the rhizosphere or the plants, promoting plant growth by mechanisms such as the biological nitrogen fixation process, or synthesis of phytohormones, improving root growth and increasing nutrient and water uptkae[1,3].

For more than a century inoculants carrying rhizobia for a variety of legumes have been commercialized worldwide[4,5]. Consequently, methods for counting rhizobial population in soil, inoculants, seeds and plants, as well as for evaluating their symbiotic performance are well established[6-8]. However, particularly in the last two decades, the use of other non-rhizobia plant-growth promoting bacteria (PGPB) in inoculants has exponentially increased worldwide[9]. Undoubtedly, the most studied and used PGPB belong to the genus *Azospirillum*, encompassing bacteria with remarkable capacity to benefit a broad range of plant species, mainly by two mechanisms, synthesis of phytohormones and biological nitrogen fixation[10-16], but that may include a surprisingly number of other biological mechanisms[3]. For example, the synthesis of signals may confer to plants tolerance of abiotic and biotic stresses[3], and the synthesis of siderophores may help in the protection against plant pathogens[1,14,17,18].

Nowadays, Brazil and Argentina are the world leaders in the use of inoculants carrying rhizobia and *Azospirillum*[9,12,19]. In Brazil, *Azospirillum* has been increasingly used in inoculants for the non-legume crops maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.)[11], pastures of brachiarias (*Urochloa* spp.)[20], and also for co-inoculation of the legumes soybean (*Glycine max* (L.) Merr.) and common bean (*Phaseolus vulgaris* L.)[21,22]. Noteworthy, the first commercial inoculant with *Azospirillum* in Brazil was launched in 2009, and reached about 7 million of commercialized doses in the last crop season[9]. About 85% of the world's agricultural grain production uses pesticides to control a variety of pests and diseases[23], and one of the main challenges for the success of microbial inoculants is the compatibility with agrochemicals used in seed treatments, especially fungicides and insecticides[4,19,24]. Low compatibility between rhizobia and pesticides has been long and broadly reported, causing sharp drop in cell survival upon contact with chemicals, decreasing nodulation and yield; therefore, seed treatment with pesticides and inoculants are often incompatible[19,24,25]. However, the use of pesticides for seed treatment continues to increase, representing a threat on the ever-growing increasing use of inoculants, which requires cautious investigation on the compatibility between microbial inoculants and pesticides. Advances in methods for investigating compatibility of rhizobia with pesticides have been achieved and are broadly used in Argentina, Brazil and Uruguay. The main method used in South America was initially developed by Penna et al. (2011)[27], for the evaluation of *Bradyrizobium*-fungicides compatibility on soybean seeds. Nowadays, several laboratories from Argentina, Brazil and Uruguay use this method, or slight modifications of it as a quality control procedure for soybean inoculants[26-29].

Azospirillum may also be affected by pesticides used in seed treatments, and the number of fungicides and pesticides used for the maize crop has increased; for example, more than 30 fungicides are registered for the control of maize diseases in Brazil[30]. However, we found no study reporting the recovery of *Azospirillum* cells from inoculated seeds of maize. There are well-established methods in the literature for the isolation and identification of endophytic and rhizospheric diazotrophic bacteria in roots, stems and leaves of non-leguminous plants[31]. However, these methods do not meet the objective of this study, which aims to count the number of viable cells surviving on the surface of inoculated seeds. First, we tried to apply the method used for the recovery and counting of *Bradyrhizobium* from soybean inoculated seeds to the *A. brasilense*-maize combination, trying to verify if this method would be applicable to different plant species and microorganisms. We were surprised to recover zero or close to zero colony forming units (CFU). As the recovery of inoculated cells from seeds is the most accurate way to confirm their ability to survive at sowing time, we performed several tests and developed a method to recover *Azospirillum* viable cells from inoculated maize seeds.

MATERIALS AND METHODS

Bacterial strains and cultivation methods

The strains used in this study were *Azospirillum brasilense* Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084), used in commercial inoculants in Brazil[11,29]. Although the great majority of the commercial inoculants in Brazil carry the two strains, we decided to study them separately, in order to observe if both responded in a similar way. The study also included *Bradyrhizobium japonicum* strain SEMIA 5079 (=CPAC 15, =CNPSo 07), used in commercial inoculants for the soybean crop in Brazil[19]. The bacteria are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Parana, Brazil.

For the evaluations, an initial inoculum of each *A. brasilense* strain was obtained in flasks containing 100 mL of liquid DYGS medium (per liter: glucose, 2 g; malic acid, 2 g; bacto-peptone, 1.5 g; yeast extract, 2g; K_2HPO_4, 0.5g; $MgSO_4 \cdot 7H_2O$, 0.5g; glutamic acid, 1.5g, pH 6.8)[32] with shaking at 120 rpm, at 28°C, for 24 h. Cell concentration was estimated by reading the optical density (O.D.) at 600 nm, and adjusted to a growth curve previously built establishing the relationship between O.D. and CFU (colony forming units) of each strain. Inocula used in the experiments were always adjusted to a cellular concentration of 10^8 CFU mL^{-1} with sterile saline solution (0.85% NaCl). For the experiment with *B. japonicum*, strain SEMIA 5079 was grown in modified-YM (yeast-mannitol) medium[33].

Cell recovery

A. brasilense cells recovery from maize seeds inoculated separately with *A. brasilense* strains Ab-V5 or Ab-V6 was first performed following the current methodology of cell recovery of *Bradyrhizobium* spp. from soybean seeds followed by the Brazilian legislation[26,29], as described in[28], but replacing the growth medium for DYGS medium for *Azospirillum*.

For each strain, 2.5 mL of each inoculant at the concentration of 10^8 CFU mL^{-1} were used to inoculate 500 g of maize seeds and left to dry for 2 h. Three biological samples of 100 seeds each were transferred to sterile Erlenmeyer flasks containing 100 mL of sterile saline solution (0.85%) with Tween 80 (0.4 mL L^{-1}). The flasks were submitted to horizontal agitation at 150 rpm for 20 min, resulting in the dilution 10^0, that was named as recovery sample. After that, serial dilutions were performed and 100µL aliquot from each dilution and each replicate were spread into a Petri's dish containing RC culture medium (*Red Congo*)[34] [per liter: malic acid 5 g; K_2HPO_4 0.5g; $MgSO_4.7H_2O$, 0.2g; NaCl 0.1g; yeast extract, 0.5g; $FeCl_3.6H_2O$, 0.015g; KOH, 4.8g; Congo red solution (0.25 g 100 mL^{-1}), 15 mL, pH 7.0], which permits the recognition of *Azospirillum* colonies on plates; they form typical red colonies. Seeds may carry several microorganisms, and to decrease the contamination vancomycin (0.1 g L^{-1}) was added to the RC medium; the methodology to recover *Bradyrhizobium* from soybean seeds also includes the same antibiotic at the same concentration[29]. For each biological replicate, three technical replicates were performed. Plates were incubated for 5 days, in the dark, at 28 ± 2°C. After this period, the CFU of each plate were evaluated, resulting in zero or close to zero CFU. To try to understand what could have led to this almost zero recovery and knowing that the cell recovery of *Bradyrhizobium* spp. from soybean seeds based on this methodology is efficient, we chose to inoculate soybean seeds with *A. brasilense* and performed the cell recovery as described above, and once again we were unsuccessful in recovering *A. brasilense* from the soybean inoculated seeds.

From the results obtained in the previous analyses, a new method to evaluate the recovery of *Azospirillum* from maize seeds was developed. In a first attempt, in order to reduce the stress of *Azospirillum* during the cell recovery stages, the maize seeds were placed in contact with distilled sterile water for 2 to 4 h before inoculation. In this procedure, each inoculum was diluted in distilled sterile water (1:2, v:v) prior to seed inoculation, in order to improve the dispersion of bacteria on the surfaces of seeds. The time between inoculation and recovery was reduced from 2 h to 30 minutes. In addition, we increased the shaking time, from 30 min to 1 h during the recovery procedure.

After inoculation of the seeds, the efficacy of cell recovery was analyzed using different

Table 1. Cellular recovery and cell counting[a] of *Azospirillum brasilense* from inoculated maize and soybean seeds. Recovery in saline solution with Tween 80

	Maize seeds		Soybean seeds
Strain	Methodology for *Bradyrhizobium* sp.	H.S.[b] 4 h; 1 h shaking	Methodology for *Bradyrhizobium* sp.
A. brasilense Ab-V5	Very low [a]	Low [a]	Very low [a]
A. brasilense Ab-V6	Very low [a]	Low [a]	Very low [a]

[a] Classification based in three biological replicates, each with three technical replicates, as follows: very low recovery: zero or close to zero CFU per 100µL of recovery sample; low recovery: up to 200 CFU per 100µL of sample; very high: >700 CFU per 100µL of recovery sample; corresponding to very low recovery: zero or close to zero CFU seed^{-1}; low recovery: up to 2000 CFU seed^{-1}; very high: >7000 CFU seed^{-1}

[b] H.S., hydrated seeds in distilled sterile water

diluents in addition to the sterile saline solution (0.85%), including distilled water and DYGS liquid medium, all three added with Tween 80 (0.4 mL L^{-1}). After shaking, a 100µL aliquots of each replicate of the dilutions were spread onto RC solid culture medium, followed by incubation for 5 days, in the dark, at 28 ± 2 °C. The CFU obtained from each diluent served as parameter to classify the recovery in: very low (zero or close to zero); low (up to 200 CFU per 100µL of recovery sample); medium (between 200 and 500 CFU per 100µL of recovery sample); high (between 500 and 700 CFU per 100µL of recovery sample); and very high (above 700 CFU per 100µL of recovery sample). The choice of levels (200, 500 and 700 CFU) was based on the results obtained.

Following, an analysis was carried out to confirm the efficacy of the developed methodology. For that, 500 g of maize seeds were treated with Ab-V5 or Ab-V6 (2.5 mL at the concentration of 10^8 CFU mL of diluent), following the developed methodology (Fig. 1). Each treatment was performed in triplicate. Inoculated seeds were maintained under controlled conditions, in the dark, with relative humidity controlled at 50 ± 5% and temperature of 25°C ± 2°C.

Cell recovery was performed after different times elapsed from inoculation: 2 h and 1, 2, 4 and 15 days of inoculation. A 100µL aliquot of the 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of each replicate was spread onto RC culture medium in Petri's dishes and after incubation for 5 days, in the dark, at 28 °C the counting of colony forming units (CFU) was performed.

Finally, we also performed a further experiment with seeds treated with the fungicide Derosal® Plus (Bayer, composed by carbendazim 250 g L^{-1} and thiram 350 g L^{-1}), applied as recommended for the maize crop (40-60 mL per 20 kg of seeds) The inoculation procedure and the cell recovery were performed as described in the previous experiment. Cell recovery was performed after 2 h and 24 h of inoculation and treatments were: control (inoculated seeds without fungicide) and inoculated seeds treated with fungicides; we did not go beyond 24 h because cell recovery and counting in the treatment with fungicide was already drastically decreased after 24 h.

Statistical analysis

Statistical analysis was performed for the quantitative results obtained in the tests of cell recovery and counting in relation to the inoculation time (up to 15 days) and regarding the treatment with pesticide. The data were submitted to the analysis of variance (ANOVA), followed by comparison of means by the Tukey's test at $p<0.05$ with the statistical software Statistica v.12.5.

RESULTS

Following the current methodology used in South America for cellular recovery of *Bradyrhizobium* spp. from soybean seeds[26-29], *A. brasilense* strains Ab-V5 and Ab-V6 were single inoculated into maize seeds and submitted to the cell recovery process. Zero or near to zero cells were recovered, being classified in the category of "very low" (Table 1), highlighting that the methodology was not suitable to evaluate the recovery of viable cells of *A. brasilense* from inoculated maize seeds.

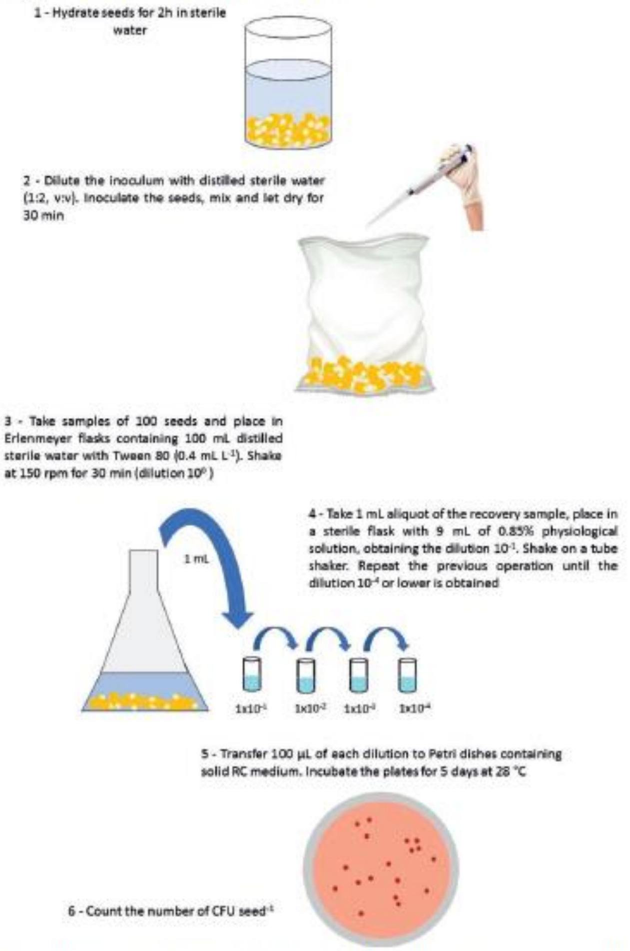
In view of the negative results, we decided to inoculate *A. brasilense* on soybean

Table 2. Cellular recovery and cell counting[a] of *Azospirillum brasilense* from inoculated maize seeds using different diluents

Strain	1 h agitation	H.S.[b] 4 h; 30 min agitation	H.S. 2 h; 30 min agitation
		Recovery in DYGS medium with Tween 80	
Ab-V5	Medium [a]	Very high [a]	Very high [a]
Ab-V6	High [a]	Very high [a]	Very high [a]
		Recovery in water with Tween 80	
Ab-V5	Very low [a]	Very high [a]	Very high [a]
Ab-V6	Low [a]	Very high [a]	Very high [a]

[a] Classification based in three biological replicates, each with three technical replicates, as follows: very low recovery: zero or close to zero CFU per 100µL of recovery sample; low recovery: up to 200 CFU per 100µL of recovery sample; medium recovery: between 200 and 500 CFU per 100 µL of recovery sample; high recovery: between 500 and 700 CFU per 100µL of recovery sample; very high: >700 CFU per 100µL of recovery sample; corresponding to very low recovery: zero or close to zero CFU seed^{-1}; low recovery: up to 2000 CFU seed^{-1}; medium recovery: between 2000 and 5000 CFU seed^{-1}; high recovery: between 5000 and 7000 CFU seed^{-1}; very high: >7000 CFU seed^{-1}.

[b] H.S., hydrated seeds in distilled sterile water.



1 - Hydrate seeds for 2h in sterile water

2 - Dilute the inoculum with distilled sterile water (1:2, v:v). Inoculate the seeds, mix and let dry for 30 min

3 - Take samples of 100 seeds and place in Erlenmeyer flasks containing 100 mL distilled sterile water with Tween 80 (0.4 mL L^{-1}). Shake at 150 rpm for 30 min (dilution 10^0)

4 - Take 1 mL aliquot of the recovery sample, place in a sterile flask with 9 mL of 0.85% physiological solution, obtaining the dilution 10^{-1}. Shake on a tube shaker. Repeat the previous operation until the dilution 10^{-4} or lower is obtained

5 - Transfer 100 µL of each dilution to Petri dishes containing solid RC medium. Incubate the plates for 5 days at 28 °C

6 - Count the number of CFU seed^{-1}

Fig. 1. Schematic outline of the method for estimating the recovery and counting of viable cells of *Azospirillum* from inoculated maize seeds.

seeds, as the recovery of *Bradyrhizobium* in soybean seeds is very successful[28]. Similar results, with lack of recovery of *Azospirillum* cells were also obtained in the analysis of soybean seeds inoculated with strains Ab-V5 and Ab-V6 (Table 1), whereas high concentration of *B. japonicum* SEMIA 5079 was obtained when the soybean seeds were inoculated with this bacterium, reaching 10^5 to 10^6 CFU seed^{-1} (data not shown). These results suggested that the inability of *A. brasilense* being recovered by this method was related to specific properties of *A. brasilense*, and not to the maize seed tegument.

As a first modification to try to improve the recovery of *A. brasilense* cells, we verified the effects of hydration of the maize seeds prior to inoculation, the reduction of the time between inoculation and cell recovery, and shaking time. These changes had positive effects on cell recovery (Table 1), but these changes in the methodology were not sufficient to obtain high recovery of cells.

Following, we checked for the efficacy of different diluents in the seed cells recovery phase, comparing saline solution, distilled water, and DYGS medium, all three with Tween 80 (0.4 mL L^{-1}), to verify which one resulted in the highest number of cells in the dilution 10^0. Both distilled water and DYGS medium resulted in higher cell recovery and counting, in comparison to the current methodology of recovery of *Bradyrhizobium* from soybean seeds with saline solution (Tables 1 and 2). When the seeds were previously hydrated in distilled water and remained under agitation for 1 h, the recovery ranged between "very low" and "low" when water was used as diluent and between "medium" and "high" when the DYGS medium was used as diluent (Table 2). When the seeds were exposed to hydration for 2 and 4 h and remained under stirring for 30 minutes, both diluents also resulted in cell recovery and counting classified as "very high".

Based on these results, the following conditions were chosen for *A. brasilense* recovery: seeds previously hydrated in distilled sterile water for 2 h, inoculum diluted in distilled sterile water prior to seed inoculation (1:2, v:v), recovery step to the dilution 10^0 in water with Tween 80 (0.4 mL L^{-1}), and shaking for 30 min. The subsequent steps, to obtain the following dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}), plating of the samples and counting of CFU followed the current methodology for recovery of *Bradyrhizobium* from soybean seeds[26-29]. The steps can be visualized in Fig. 1.

In order to confirm the efficacy of the developed methodology, we performed the cellular recovery of *A. brasilense* from maize seeds using the new proposed methodology at different times of inoculation (2 h and 1, 2, 4 and 15 days of inoculation). The recovery of *Azospirillum* viable cells from maize seeds was estimated at 3.84 x 10^5 CFU seed^{-1} for Ab-V5 and at 5.07 x 10^5 CFU seed^{-1} for Ab-V6 with 2 h of inoculation (Fig. 2). As expected, the number of viable cells recovered from the seeds decreased with time, reaching zero for Ab-V5 and 3.33 CFU seed^{-1} for Ab-V6 after 15 days (Fig. 2). In general, the two strains showed similar behavior, with differences detected only after one day (Fig. 2).

Our last analysis consisted of the recovery of *A. brasilense* Ab-V5 and Ab-V6 strains from maize seeds previously treated or not with fungicide. After 2 h of inoculation, the concentration of recovered cells from the seeds without fungicideswas (SD in parenthesis) of 1.96 x 10^5 CFU seed^{-1} (±4.1 x 10^4) for Ab-V5 and 1.55 x 10^5 CFU seed^{-1} (±3.63 x 10^4) for Ab-V6. Cell recovery from seeds previously treated with Derosal® Plus fungicide was 3.37 x 10^5 CFU seed^{-1} (±2.11 x 10^4) for Ab-V5, differing significantly from the control, and 5.11 x 10^5 CFU seed^{-1} (±2.3 x 10^5) for Ab-V6. In fungicide-treated seeds the cell concentration

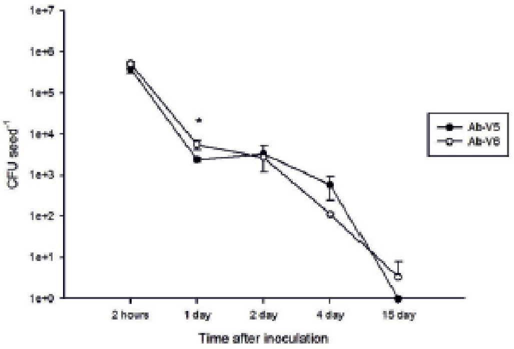


Fig. 2. Concentration of viable cells of *Azospirillum brasilense* recovered from maize seeds after different periods of inoculation. The data represent the means of three replicates and the bars denote the standard error. (*) treatments that differ from each other by the Tukey's test at $p < 0.05$; without (*) indicates that values were not statistically different.

was drastically reduced with 24 h of inoculation, reaching zero CFU recovered at dilution 10^{-1}, whereas in the seeds not treated with fungicides the concentration of recovered cells was of 6.55 x 10^2 CFU $seed^{-1}$ (±8.31 x 10^1) for Ab-V5 and of 3.55 x 10^2 CFU $seed^{-1}$ (±9.54 x 10^1) for Ab-V6.

DISCUSSION

Brazil, Argentina and Uruguay are well known inoculant consumers, amounting to an inoculant marked nowadays of over 120 million doses year. The method adopted in these countries for the recovery of *Bradyrhizobium* cells from inoculated soybean seeds has been broadly and successfully used[26-29]. The method is also applicable to other plant-microbe combinations, such as common bean and *Rhizobium tropici* (data not shown); in addition, dozens of studies, including reports by our group[13] have successfully recovered *Azospirillum* and other diazotrophic bacteria from maize rhizosphere, roots and leaves. Therefore, we were surprised for not being able to recover viable cells from maize seeds inoculated with *Azospirillum*. The same results were obtained when *A. brasilense* was inoculated on soybean seeds, while *Bradyrhizobium* cells inoculated on soybean seeds had a successful recovery.

In view of all the proven benefits of inoculation of maize seeds with *A. brasilense* described in detail in the literature[1,3,10,14,15,17,18], it is very unlikely that the cells inoculated and not recovered are not viable. Therefore, we believe that the tested methodology was not applicable. As *A. brasilense* is able to colonize the interior of plant tissues[14], one hypothesis for the almost zero recovery result could be that the bacterium, when in contact with the seed surface, would invade the plant tissue. However, it is not yet known in what situations and how long it would take for *A. brasilense* cells to leave the seed surface and start invading the cells; in addition, the species establishes preferentially in the rhizosphere.

A well-known feature of *A. brasilense* is its ability to aggregate and flocculate when exposed to various stressing conditions[14, 35-39]. Despite several studies showing that the aggregation of *A. brasilense* increases its tolerance to desiccation and high temperatures (40 °C), positively affecting its survival[40,41], this represents a serious limitation in studies to verify the survival of *Azospirillum* on seeds at the sowing time, including the compatibility with pesticides, pre-inoculation, among others.

Therefore, another hypothesis for the unsuccessful recovery was that the contact of *A. brasilense* with seeds, a dry and nutritionally poor surface, would result in bacteria aggregation/flocculation. Consequently, when inoculated in solidified culture medium, each cell aggregate, containing hundreds of cells, would result in only one CFU, underestimating the number of CFU $seed^{-1}$. Based on this hypothesis, alternatives were investigated to reduce the stressing conditions. First, we investigated the effect of seed hydration with sterile distilled water for up to 4 h before inoculation, as well as the effect of increasing the shaking time. When maize seeds were hydrated prior to inoculation, the conditions were less stressful to the bacteria, reducing the cell aggregation/flocculation, and resulting in satisfactory recovery, while increasing the shaking time from 30 min to 1 h did not impact the recovery of both strains (Tables 1 and 2). It is important to consider that this is a methodology for laboratory analysis of the quality of inoculants, so the seeds used for this purpose would not be taken to the field by farmers. One may also consider that the absorption kinetics of the inoculant might be affected by the pre-treatment. However, the volume used was considerably lower than that usually employed by the farmers in liquid products used for seed treatment and, in addition, in our methodology we investigated the minimum volume needed to allow cell recovery and counting.

In this new methodology of recovering cells of *Azospirillum* from inoculated seeds, the first step consists in effectively removing the inoculated bacteria from the seeds and to transfer them to an aqueous medium for further analysis of cell counting. Saline solution (NaCl 0.85%) is used as a diluent in most of the microbiological techniques, acting on the osmotic balance between inside the cell and the external environment. However, as the methodology using saline solution failed, we proceeded to a second modification that consisted in replacing the saline solution by different diluents, including distilled water and DYGS medium, always with Tween 80. The two new diluents positively influenced the bacterial cellular recovery from the seeds and cell counting. Considering these results, we hypothesized that the saline solution would also contribute to the cellular aggregation/flocculation, interfering in the final counting of CFU. As both distilled water and DYGS medium presented similar results, we opted by distilled water with Tween 80, due to the low cost and easy preparation compared to the DYGS medium. Therefore, we may conclude that by hydrating the seeds before inoculation and by using distilled water with Tween 80 as a diluent for cell recovery, the stressing conditions for *Azospirillum* will be reduced, decreasing aggregation/flocculation and allowing proper cell recovery and counting.

In general, quality control of microbial inoculants is based on cell concentration and absence of contaminants. Great progresses have been achieved at the industry, with the development of products with high concentration of cells, while legal or private control of the concentration guarantees that the farmers will receive appropriate products[4, 8, 19, 42, 43]. However, even more important than inoculant concentration is to know how many cells are alive on the inoculated seeds at sowing. In this context, the proposed method is of great relevance for the evaluation of the efficacy of inoculation of maize seeds, because it allows to know the cellular concentration of *A. brasilense* in each inoculated seed. This information is very important and our results for cell recovery after inoculation and in seeds treated with fungicides show that it is useful in several studies, such as the development of new inoculant formulations, verification of compatibility between pesticides and inoculants, viability of pre-inoculation, among others, and may also help to guide policies of recommendation of microbial inoculants, so that the farmers will benefit from the best technologies.

CONCLUSION

A methodology was developed to evaluate the recovery and counting of *Azospirillum* viable cells from inoculated maize seeds (Fig. 1). The methodology was successful to recover and count viable cells of *A. brasilense* from maize seeds after different times of inoculation, as well as to verify the effects of pesticides on *Azospirillum* survival on the seeds, and may as well be applied to several other studies related to inoculants and inoculation.

AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: MSS, MM, MH, Performed the experiments: MSS, TFR, EF. Analyzed the data: MSS, TFR, EF. MM, MAN, MH. Contributed reagents/materials/analysis tools: MAN, MH. Wrote the paper: MSS, TFR, EF, MM, MAN, MH

All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The authors declare no ethical conflicts; authors declare that they have consented to participate in the manuscript and publish it.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript, and complementary dataset will be available upon request to the corresponding author

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CAPÍTULO 4

COMPATIBILIDADE DE *Azospirillum brasilense* COM AGROTÓXICOS UTILIZADOS NO TRATAMENTO DE SEMENTES DE MILHO

COMPATIBILIDADE DE *Azospirillum brasilense* COM AGROTÓXICOS UTILIZADOS NO TRATAMENTO DE SEMENTES DE MILHO

RESUMO

O tratamento de sementes com agrotóxicos é comumente usado como procedimento inicial de proteção de plantas contra pragas e doenças. No entanto, o uso de tais produtos químicos pode prejudicar a sobrevivência e o desempenho de microrganismos benéficos introduzidos via inoculação, como a bactéria *Azospirillum brasilense*, promotora de crescimento de plantas. Neste estudo, foi avaliada a compatibilidade entre o agrotóxico mais comumente utilizado no Brasil para o tratamento de sementes de milho, composto por dois fungicidas e um inseticida, com as estirpes comerciais Ab-V5 e Ab-V6 de *A. brasilense*, e os consequentes impactos nas desenvolvimento de plantas. A toxicidade do agrotóxico para *A. brasilense* foi confirmada, com aumento da mortalidade celular após apenas 24 h de exposição *in vitro*. A germinação das sementes e o crescimento das plântulas não foram afetados por *A. brasilense*, nem pelo agrotóxico. Entretanto, em casa de vegetação, o agrotóxico afetou negativamente o volume radicular, a massa radicular seca e a incidência de pelos radiculares, mas a toxicidade foi atenuada pela inoculação com *A. brasilense* para os parâmetros de volume e incidência de pelos radiculares. Em sementes de milho inoculadas com *A. brasilense*, o agrotóxico afetou negativamente o número de ramificações das raízes, a incidência de pelos radiculares e o comprimento de pelos radiculares. Consequentemente, novas formulações de inoculantes com protetores celulares e o desenvolvimento de agrotóxicos compatíveis devem ser pesquisados para garantir os benefícios da inoculação com bactérias promotoras de crescimento de plantas.

Palavras-chave: Inoculação, compatibilidade, desenvolvimento de raizes, agrotóxicos, *Azospirillum*.

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Research Article

Compatibility of *Azospirillum brasilense* with Pesticides Used for Treatment of Maize Seeds

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Seed treatment with chemical pesticides is commonly used as an initial plant protection procedure against pests and diseases. However, the use of such chemicals may impair the survival and performance of beneficial microorganisms introduced via inoculants, such as the plant growth-promoting bacterium *Azospirillum brasilense*. We assessed the compatibility between the most common pesticide used in Brazil for the treatment of maize seeds, composed of two fungicides, and one insecticide, with the commercial strains Ab-V5 and Ab-V6 of *A. brasilense*, and evaluated the impacts on initial plant development. The toxicity of the pesticide to *A. brasilense* was confirmed, with an increase in cell mortality after only 24 hours of exposure *in vitro*. Seed germination and seedling growth were not affected neither by the *A. brasilense* nor by the pesticide. However, under greenhouse conditions, the pesticide affected root volume and dry weight and root-hair incidence, but the toxicity was alleviated by the inoculation with *A. brasilense* for the root volume and root-hair incidence parameters. In maize seeds inoculated with *A. brasilense*, the pesticide negatively affected the number of branches, root-hair incidence, and root-hair length. Therefore, new inoculant formulations with cell protectors and the development of compatible pesticides should be searched to guarantee the benefits of inoculation with plant growth-promoting bacteria.

1. Introduction

Global population reached 7.6 billion people in 2017 and, according to the predictions, will increase to 11.2 billion by 2100 [1]. In this context, it is necessary to produce more food but also to search for production strategies resulting in minimum environmental impact. The inoculation of crops with plant growth-promoting bacteria (PGPB), especially those that can contribute to the reduction of chemical fertilizers usage, attends to these concepts of agricultural sustainability [2].

Several benefits have been attributed to the inoculation with *A. brasilense*, including the supply of N by the biological nitrogen fixation (BNF) process [3, 4], stimulation of root growth [5, 6], phosphate solubilization [7], and increased tolerance to abiotic [8, 9] and biotic [10] stresses. In the case of the Brazilian commercial strains of *A. brasilense* Ab-V5 and Ab-V6, the main effects have been attributed to the production of phytohormones [3, 4].

The use of fungicides and insecticides for seed treatment has been broadly employed in Brazil, with maize representing the second crop that receives more pesticides, after soybean (*Glycine max* (L.) Merr.) [11]. However, the use of inoculants with *A. brasilense* for the maize crop is also impressively increasing, for example, in Brazil, from zero to 7 million doses per year^{-1} in less than a decade, and agrochemicals could affect bacterial survival and plant growth. For example, in the *Bradyrhizobium*-soybean symbiosis, the incompatibility with pesticides may cause lower nodulation and, consequently, lower BNF rates [12, 13].

There is still little information about the effects of pesticides used for the treatment of maize seeds on *A.*

brasilense survival and plant growth. As seed treatment is widespread in the maize cropping, studies on the impact of pesticides in *A. brasilense* are necessary to understand the effects on the bacterium and on the mechanisms of plant growth promotion.

2. Materials and Methods

2.1. Microorganisms, Fungicides, and Insecticides. The experiments were performed with *Azospirillum brasilense* strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084). The strains are deposited at the "Diazotrophic and Plant Growth-Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection #1213, WDCM Collection #1054) in Londrina, State of Paraná, Brazil. These two strains have been broadly used in the production of commercial inoculants for the maize crop in Brazil since 2009 [3]. Depending on the analysis, the strains were cultured together or separately.

The pesticide used was Standak™ Top (BASF), broadly employed for the treatment of maize seeds. It consists of a mixture of one insecticide (Fipronil 25%) and two fungicides (pyraclostrobin 2.5% and thiophanate-methyl 22.5%). The recommended dose is of 2 mL per kg of maize seeds.

2.2. Recovery of A. brasilense from Maize Seeds Treated with Pesticides. The recovery of *A. brasilense* strains Ab-V5 and Ab-V6 from maize seeds treated with the pesticide was based on the method described by Santos et al. [14]. The inoculum was prepared in the modified liquid DYGS medium (composed of glucose, 2 g; malic acid, 2 g; Bacto peptone, 1.5 g; yeast extract, 2 g; K_2HPO_4, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; and glutamic acid, 1.5 g; pH 6.8), incubated at 28°C, and shook at 120 rpm until the cell concentration of 10^8 CFU·mL^{-1}. Seeds were previously hydrated in distilled sterile water for 2 h and treated with Standak™ Top at the dose of 2 mL·kg^{-1}, or left untreated; both treatments were left to dry at room temperature for 2 h. The inocula were diluted in distilled sterile water prior to seed inoculation (1 : 2, v/v). Treated and nontreated seeds were then inoculated with *A. brasilense* strains Ab-V5 and Ab-V6, applied to the seeds at the rate of 5 mL·kg^{-1}, corresponding to 10^5 cells seed^{-1}. Cell recovery from seeds was performed 30 minutes and 24 h after inoculation.

Three biological samples of 100 seeds each were transferred to sterile Erlenmeyer flasks containing 100 mL of sterile water with Tween 80 (0.4 mL·L^{-1}). The flasks were submitted to horizontal agitation at 150 rpm for 30 min, resulting in the dilution 10^0. One-milliliter aliquot of the recovery sample was then transferred to a sterile flask with 9 mL of 0.85% physiological solution, resulting in the 10^{-1} dilution. The flask was shaken on a vortex. The process was repeated until the 10^{-4} dilution was obtained. 100 μL aliquot of each replicate of each dilution was spread into Rojo Congo (RC) solid culture medium [15] [composed of, per L, DL-malic acid, 5 g; yeast extract, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; NaCl, 0.1 g; KOH, 4.8 g; $FeCl_3.6H_2O$, 0.015 g; Congo Red, 15 mL (solution at 0.25 mg 100 mL); and agar, 20 g; pH 7.0], with vancomycin (0.1 g·L^{-1}) to avoid seeds' contaminants, followed by incubation for 5 days at 28 ± 2°C. The recovery of viable cells was based on the counting of colony-forming units (CFU). Each treatment was performed in triplicate.

The experiment was performed on a completely randomized design (CRD), and the data were submitted to the analysis of variance (ANOVA), followed by the comparison of means by Tukey's test at $p < 0.05$ with the statistical software.

2.3. Seed Germination and Seedling Vigor of Maize Treated with Pesticides and Inoculated with A. brasilense. In order to evaluate the compatibility between the pesticide and the *A. brasilense* inoculant, as well as the effects on seed germination and initial growth of maize seedlings, maize seeds (Agroceres AG9010 PRO VT) were surface-disinfected and, depending on the treatment, were treated or not with Standak™ Top and with a mixture of strains Ab-V5 and Ab-V6 of *A. brasilense*. Four treatments were evaluated: NPNI (control with no pesticide, no inoculant); WPNI (with pesticide, no inoculant); NPWI (no pesticide, with inoculant); and WPWI (with pesticide, with inoculant). Each treatment consisted of three replicates, each with 12 seeds and the experiment was performed in a completely randomized design (CRD).

Seed surface disinfection was performed in ethanol 70% for 1 min, hypochlorite 10% for 5 min and then washed in sterile distilled water five times. First, the seeds of the treatments with the pesticide were treated with Standak™ Top at a rate of 2 mL·kg^{-1} of seeds, as recommended by the manufacturer. Seeds were allowed to dry at room temperature for 2 h.

For each treatment, a tray was disinfected with 10% hypochlorite and then left under laminar flow UV light for 20 min. Following, three Germitest papers (JProlab®) previously autoclaved were placed in the tray and moistened with sterile water. The seeds were scattered over two papers, forming 12 columns. Following, the seeds of the treatments with inoculation each received the inoculant of *A. brasilense* at a final concentration of 10^5 cells of *A. brasilense* per seed and the third paper was placed over the seeds. The papers were rolled up; their ends were tied with elastic bands and incubated in a seed germinator DeLeo® at 25°C and 45 ± 5% of relative air humidity for seven days.

After this period, seed germination rate and seedling vigor parameters, including total length of the seedlings (from the tip of the primary root to the top of the primary leaf), the length of the shoot, and the length of the main root axis, were evaluated with the aid of a ruler with a graduation in mm [16]. The average length was obtained by summing the measurements taken from each normal seedling at each repetition and then dividing by the number of normal seedlings measured.

The experiment was performed in a completely randomized design (CRD) with the treatments arranged in a factorial scheme (2 × 2), involving presence/absence of pesticide and presence/absence of inoculation. Statistical analysis was performed using the RBIO statistical software. Data obtained were evaluated for normality and variance homogeneity, followed by the analysis of variance (ANOVA) by Tukey's test at 5% probability.

2.4. Greenhouse Experiment. A greenhouse experiment was carried out to assess the effects of the pesticide used together with the inoculant on the initial development and root morphology of maize. Plants were grown in modified Leonard jars [17] containing 750 cm^3 of sterilized substrate, consisting of a mixture of sand and pulverized coal (3 : 1, v/v), containing sterile nutrient solution with all macronutrients and micronutrients [18]. The experiment consisted of the same four different treatments described before: NPNI, WPNI, NPWI, and WPWI. The experimental design was performed in a completely randomized design (CRD) with treatments arranged in a factorial scheme (2 × 2), involving presence/absence of pesticide and presence/absence of inoculation with *A. brasilense*, with five replicates.

Maize seeds (Agroceres AG-9010) were surface-sterilized with 70% ethanol and 3% sodium hypochlorite [17]. For the treatments WPNI and WPWI, the seeds were treated with Standak™ Top (2 mL per kg of maize seeds) and left to dry for two hours. For the treatments NPNI and NPWI, this step was not performed. The seeds of the treatments NPWI and WPWI were inoculated with a mixture of Ab-V5 and Ab-V6, ensuring a concentration of 10^5 cells $seed^{-1}$. Three seeds were sown and thinned to one plant per jar four days after emergence. Nutrient solution was applied as needed, and the temperature and humidity at the greenhouse were controlled by means of air conditioners (25° ± 5°C and 80 ± 5%, respectively).

Thirty-eight days after emergence, plant height (cm) and culm diameter (mm) were assessed with the aid of a ruler and digital caliper. Plants were harvested and the shoots were separated from the roots. The shoots were oven-dried at 60°C until constant dry weight. The roots were washed with running water until completely clean. Approximately, 1 g of fresh roots from each sample was separated and stained in methylene blue (1%) solution for 1 min and washed again in water and scanned with Epson Perfection V370 Photo® for further morphological analysis. The remaining roots were oven-dried under the same conditions as the shoot.

The scanned root images were analyzed using GiA Roots® software to assess specific length ($m{\cdot}g^{-1}$), weighted average diameter (mm), tissue density, and volume (cm^3). The value determined in each scanned root fragment was estimated for the total fresh mass of the root system.

Approximately, 0.1 g of fresh fine roots was obtained from each sample, stored in FAA solution (90% ethanol 70%, 5% formaldehyde, and 5% acetic acid) and used for assessment of root-hair incidence, root-hair length, and number of root branches. The number of root branches was counted using a stereomicroscope at 30X magnification to estimate the number of lateral roots. Root-hair incidence was determined by the presence or absence of root hairs on 150 fine-root intersections using the gridline method [19]. Root-hair length was assessed measuring 50 root hairs in fine-root segments using a microscope at 100X magnification with an ocular micrometer.

The dataset was first evaluated for normality and variance homogeneity. Means were compared using the analysis of variance (ANOVA) followed by Tukey's test at 5% probability. All analyses were performed in the software RBIO®.

3. Results and Discussion

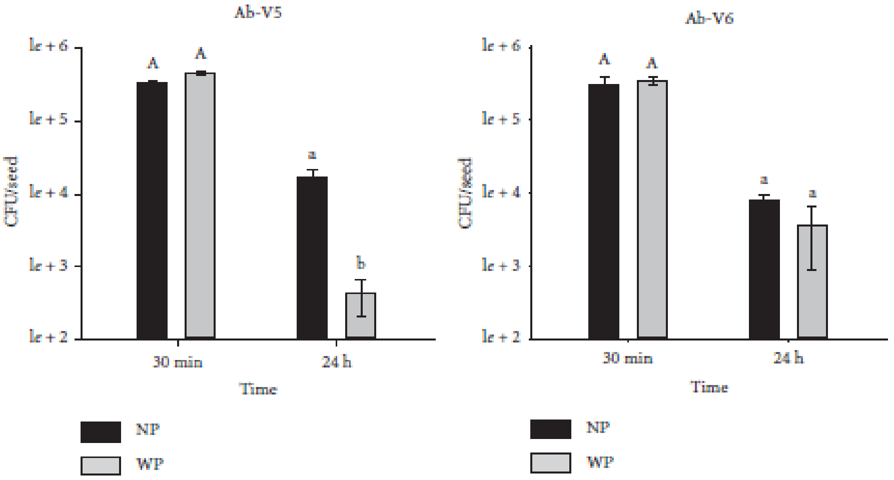
The number of *A. brasilense* cells recovered from maize seeds significantly decreased after 24 h of inoculation for both strains, with or without pesticide (Figure 1). In addition, strain Ab-V5, but not Ab-V6, was significantly affected by the seed treatment with the pesticide after 24 h, with a drastic decrease from 4.56×10^5 CFU $seed^{-1}$ in the day of inoculation to 4.37×10^2 CFU $seed^{-1}$ after 24 h. Santos et al. [14] also reported drastic cell mortality of *A. brasilense* after 24 h of exposure to another pesticide (carbendazim + thiram). Pereira et al. [20] observed similar results when exposing these same strains to pesticide-coated (metalaxyl-m + fludioxonil + thiamethoxam + abamectin + Peridiam) maize seeds; after 12 h of exposure, the authors observed a cell survival rate of 65.87% in seeds without pesticide and of 13.56% when treated with the pesticide.

It is important to comment that farmers usually treat their seeds with pesticides and inoculants and sowing may take several hours or even days. Our results indicate that *A. brasilense* is a very sensitive bacterium, with poor survival capacity in maize seed surface, even in the absence of pesticides. Therefore, cell protectors in inoculant formulations, speed of sowing, and other strategies should be searched to guarantee the performance of elite strains of *A. brasilense*.

Neither the inoculation of *A. brasilense* nor the seed treatment with the pesticide affected significantly seed germination rate, ranging from 86.10 to 91.66% (Table 1). Similar results were observed by Vogel and Fey [21]. Dartora et al. [22] also found no effect of the fungicide fludioxonil-metalaxyl and of *A. brasilense* on the percentage of germination of maize seeds. Similarly, in wheat (*Triticum aestivum*), Muraneto et al. [23] observed no differences in germination of seeds treated with fungicide, insecticide, and *A. brasilense*.

For the seed vigor parameters, there was also no interaction between the factors (pesticide and inoculation). However, the best results for total length (36.93 cm), root length (22.66 cm), and shoot length (13.26 cm) were observed in the treatment without pesticide and inoculation (NPWI) treatment. Some farmers have concerns about the inoculation with *Azospirillum* affecting seed germination and seedling growth. Therefore, our data confirm that there was no negative effect caused by strains Ab-V5 and Ab-V6 that could affect plant emergence.

Plant height varied between 67 and 71 cm, culm diameter between 9.6 and 10.7 mm, and shoot dry weight between 2.5 and 3 g, with no significant interaction between the inoculation and the pesticide for any of the parameters (Table 2). Although without statistical difference, the lowest height,



(a)

(b)

Figure 1: Recovery of *Azospirillum brasilense* cells (expressed in CFU $seed^{-1}$) from maize seeds treated (WP) or not (NP) with pesticide 30 minutes and 24 h after inoculation. Data represent the means of three biological replicates, each with three technical replicates ($n = 9$), and when followed by the same letter do not differ from each other by Tukey's test at $p < 0.05$. (a) Ab-V5; (b) Ab-V6.

Table 1: Germination rate and initial growth of maize seedlings whose seeds were treated or not with pesticide and were inoculated or not with *Azospirillum brasilense* strains Ab-V5 and Ab-V6.

Treatments	Germination rate (%)	Total length (cm)	Root length (cm)	Shoot length (cm)
NPNI	88.88 Aa	33.34 Aa	21.42 Aa	10.91 Aa
WPNI	88.88 Aa	34.15 Aa	20.85 Aa	12.30 Aa
NPWI	86.10 Aa	36.93 Aa	22.66 Aa	13.26 Aa
WPWI	91.66 Aa	35.03 Aa	21.80 Aa	12.23 Aa
p-value	0.24	0.90	0.47	0.84
CV (%)	14.06	4.41	4.86	8.97

Data represent the means of five replicates and when followed by the same letter do not differ from each other by Tukey's test at $p < 0.05$. Uppercase letters compare means within the same pesticide condition (WP: with pesticide and NP: no pesticide) and lowercase letters compare means within the same inoculation condition (WI: with inoculation and NI: no inoculation).

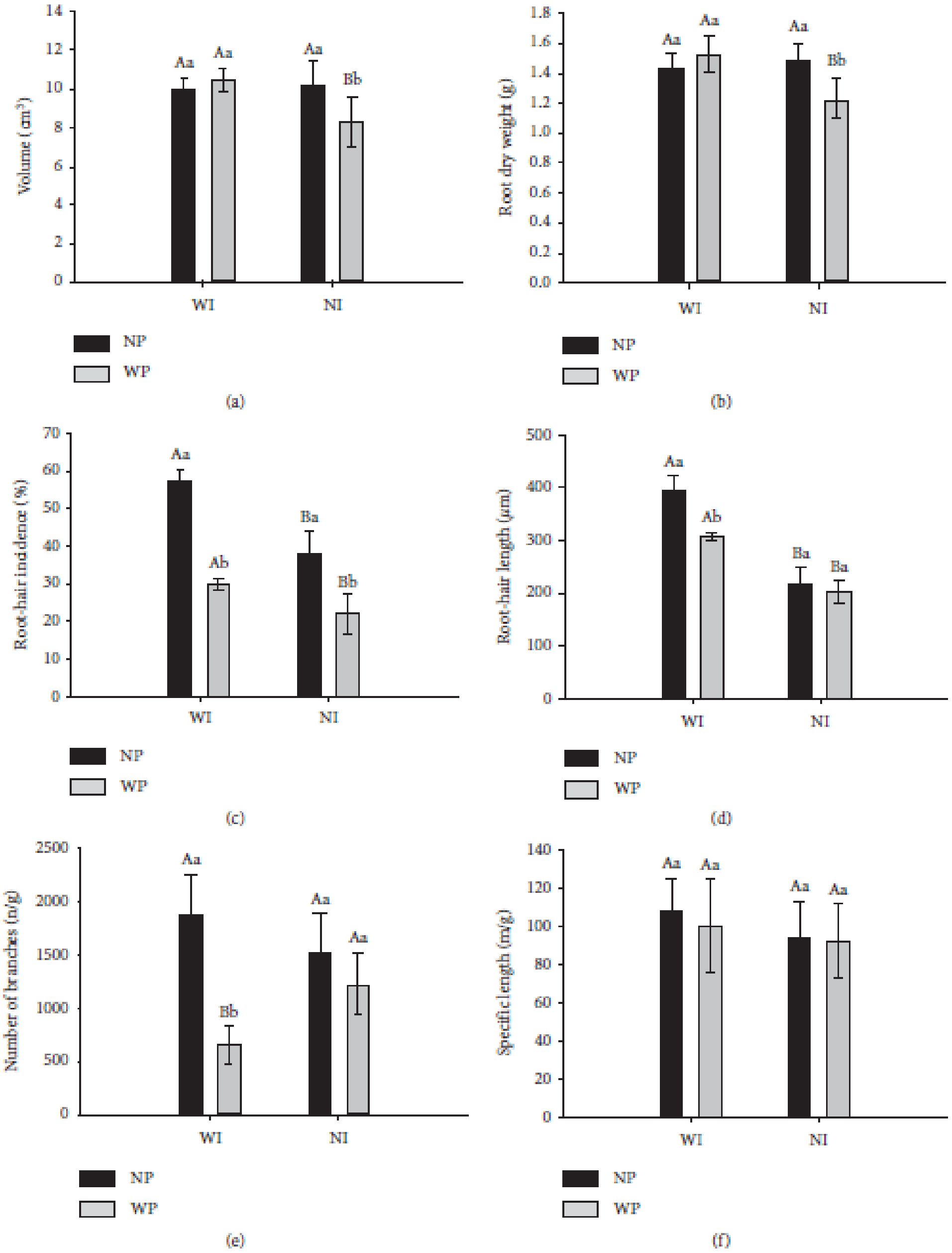
Table 2: Shoot attributes of maize whose seeds were treated or not with pesticide and were inoculated or not with *Azospirillum brasilense* strains Ab-V5 and Ab-V6.

Treatments	Plant height (cm)	Culm diameter (mm)	Shoot dry mass (g)
NPNI	71.0 Aa	10.4 Aa	3.0 Aa
WPNI	67.0 Aa	9.6 Aa	2.5 Aa
NPWI	67.7 Aa	10.0 Aa	2.7 Aa
WPWI	71.0 Aa	10.7 Aa	2.8 Aa
p-value	0.702	0.835	0.0867
CV (%)	5.94%	9.82%	12.84%

Means of five replicates followed by the same letter do not differ from each other by Tukey's test at $p < 0.05$. Uppercase letters compare means within the same pesticide condition (WP: with pesticide and NP: no pesticide) and lowercase letters compare means within the same inoculation condition (WI: with inoculation and NI: no inoculation).

culm diameter, and shoot dry mass were observed in the treatment with pesticide and no inoculation (WPNI).

Even when noninoculated maize seeds were treated with the pesticide, there was a decrease in root volume (Figure 2(a)), root dry weight (Figure 2(b)), and root-hair number (Figure 2(c)); therefore, the stress caused in plants by pesticides can reduce the efficiency of the root system in acquiring water and nutrient resources and, consequently, plant growth. Bonea and Bonciu [24] investigated the cytological and genotoxic effects induced by the fungicide Royal Flo, with thiram in its composition, and verified that the fungicide reduced the mitotic index of the root meristem.

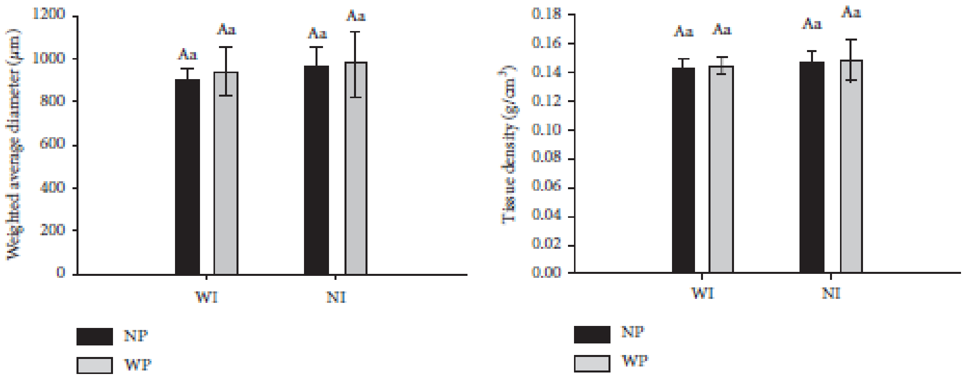


(a) (b)

(c) (d)

(e) (f)

Figure 2: Continued.



(g) (h)

Figure 2: (a) Volume; (b) root dry weight; (c) root hair incidence; (d) root hair length; (e) number of branches; (f) specific length; (g) weighted average diameter; and (h) tissue density of roots of maize whose seeds were treated or not with pesticide and were inoculated or not with *Azospirillum brasilense* strains Ab-V5 + Ab-V6. Plants were grown under greenhouse conditions for 38 days. Data represent the means of five replicates ($n = 5$) and when followed by the same letter do not differ from each other by Tukey's test at $p < 0.05$. Uppercase letters compare means within the same pesticide condition (WP: with pesticide and NP: no pesticide) and lowercase letters compare means within the same inoculation condition (WI: with inoculation and NI: no inoculation). Vertical bars represent the standard deviation.

In our study, we showed that inoculation with *A. brasilense* helped to mitigate the toxic effects caused by the pesticide on root volume and root dry weight.

The main mechanism by which strains Ab-V5 and Ab-V6 of *A. brasilense* promote plant growth is by the production of auxins that stimulate root development [4, 9]. The auxins are important modulators of cell division, acting on the differentiation and elongation of the root apex, on the initiation and development of secondary roots, and on the development of the vascular system [25]. Indeed, inoculation with *Azospirillum* leads to lateral root promotion [26], helping plant development and increasing the ability to acquire water and mineral nutrients from soil [27]. One of the most pronounced effects of *Azospirillum* inoculation on root morphology is the proliferation of root hairs, thin extensions of root epidermal cells that occur mainly near the apexes of stretching roots [27, 28]. In our study, considering the seeds not treated with the pesticide, the main impact of inoculation with *A. brasilense* was on root-hair incidence (Figure 2(c)) and root-hair length (Figure 2(d)), which are very important properties for the uptake of water and nutrients [27]. However, in the presence of the pesticide, the benefits of inoculation were impaired for these two parameters and also for the number of branches per gram of root (Figure 2(e)).

The increase of root-hair incidence due to inoculation with *Azospirillum* sp. has been reported in other cultures as pearl millet (*Pennisetum americanum* L.) [29], wheat [30, 31], rice (*Oryza sativa* cv.) [32], burr medic (*Medicago polymorpha* L.) [33], alfalfa (*Medicago sativa*) [34], and tomato (*Lycopersicon esculentum* Mill.) [35, 36].

Recently, the impact of inoculation with *A. brasilense* has also been described by Rondina et al. [37] who studied the morphology of soybean roots inoculated with different treatments and observed that inoculation with *Bradyrhizobium* spp. together with *A. brasilense* (Ab-V5 and Ab-V6) increased specific root length, root-hair length, and the number of root branches compared to the single inoculation with *Bradyrhizobium* spp. The authors also reported that the presence of *A. brasilense* inoculated together with *Bradyrhizobium* spp. was determinant for the increase in the percentage of root length with diameter <0.50 mm.

Facing the benefits that can be achieved by the inoculation with *A. brasilense*, particularly with strains Ab-V5 and Ab-V6 [3, 4, 9, 28, 38], nowadays used in more than 7 million doses of inoculants commercialized per year in Brazil, it was mandatory to verify if the treatment of maize seeds with pesticides, broadly adopted by the farmers (e.g., [11]), was compatible with the bacteria. Some of the root parameters evaluated, such as specific length (Figure 2(f)), weighted average diameter (Figure 2(g)), and tissue density (Figure 2(h)), were not affected by the pesticide, neither in inoculated nor in noninoculated plants. However, other parameters such as root-hair incidence (Figure 2(c)), root volume (Figure 2(a)), and root dry weight (Figure 2(b)) were decreased even in noninoculated plants. Noteworthy, for these last two parameters, the inhibition caused by the pesticide was alleviated by the inoculation with *A. brasilense*, highlighting that the bacterium may play a key role in the mitigation of the abiotic stress, as pointed out before for other abiotic stresses, such as salinity [4, 9, 36].

In addition, although we did not find differences in shoot parameters at this early evaluation of plant growth, we should consider that plants were growing under optimal controlled conditions of greenhouse, and that on the field the increase in number of hair and root-hair length in inoculated plants may both decrease the susceptibility to water stress conditions and improve maize nutrition, achieving good productivity results. This is even more important with the increasing number of episodes of drought reported with the global climate change.

4. Conclusions

We observed lower survival of *A. brasilense* in the presence of the pesticide Standak™ Top, with an increase in cell mortality after only 24 h of exposure. The treatment of maize seeds with Standak™ Top conferred toxic effects on plants, interfering with their development. Consequently, the known benefits of maize inoculation with *A. brasilense* (e.g., [3, 9, 28]), especially on cell division, differentiation, and elongation of roots, may be impaired by the seed treatment with pesticides. It is important to search for innovative inoculants containing cell protectors (e.g., [39, 40]), or to develop more compatible pesticides, so that the expected benefits of inoculation with plant growth-promoting bacteria can be obtained.

Data Availability

The data are included in the manuscript. Data with all replicates can be available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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CAPÍTULO 5

O DESAFIO DE COMBINAR ALTOS RENDIMENTOS COM BIOPRODUTOS AMBIENTALMENTE AMIGÁVEIS: UMA REVISÃO SOBRE A COMPATIBILIDADE DE PESTICIDAS COM INOCULANTES MICROBIANOS

O DESAFIO DE COMBINAR ALTOS RENDIMENTOS COM BIOPRODUTOS AMBIENTALMENTE AMIGÁVEIS: UMA REVISÃO SOBRE A COMPATIBILIDADE DE PESTICIDAS COM INOCULANTES MICROBIANOS

RESUMO

Inoculantes ou biofertilizantes com o objetivo de substituir parcial ou totalmente os fertilizantes químicos estão se tornando cada vez mais importantes na agricultura, pois há uma percepção global da necessidade de aumentar a sustentabilidade. Nesta revisão, discutimos alguns resultados importantes da inoculação de uma variedade de culturas com rizóbios e outras bactérias promotoras de crescimento de plantas (BPCP). Melhorias importantes na qualidade dos inoculantes e na identificação de novas estirpes e formulações foram alcançadas. No entanto, a agricultura continuará a demandar agrotóxicos e sua baixa compatibilidade com inoculantes, principalmente quando aplicados via sementes, representa uma grande limitação para o sucesso da inoculação. As diferenças na compatibilidade entre agrotóxicos e inoculantes dependem de seu princípio ativo, formulação, época de aplicação e tempo de contato com os microrganismos; entretanto, em geral, eles têm um alto impacto na sobrevivência e no metabolismo das células microbianas, afetando sua contribuição para o crescimento das plantas. São necessárias novas estratégias para superar o problema da incompatibilidade entre agrotóxicos e inoculantes, pois as que foram propostas até agora ainda são muito modestas quanto ao cenário atual de uso de agrotóxicos nas culturas.

Palavras-chave: rizóbios, bactérias promotoras de crescimento de plantas, fungicida, inseticida, herbicida, fixação biológica de nitrogênio, inoculação

*Review – **Agronomy***

The challenge of combining high yields with environmentally-friendly bioproducts: A review on the compatibility of pesticides with microbial inoculants

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The challenge of combining high yields with environmentally-friendly bioproducts: A review on the compatibility of pesticides with microbial inoculants

ABSTRACT: Inoculants or biofertilizers aiming to partially or fully replace chemical fertilizers are becoming increasingly important in agriculture, as there is a global perception on the need to increase sustainability. In this review, we discuss some important results of inoculation of a variety of crops with rhizobia and other plant growth-promoting bacteria (PGPB). Important improvements in the quality of the inoculants and on the release of new strains and formulations have been achieved. However, agriculture will continue to demand chemical pesticides, and their low compatibility with inoculants, especially when applied to seeds, represents a major limitation to the success of inoculation. The differences in the compatibility between pesticides and inoculants depend on their active principle, formulation, time of application, and period of contact with living microorganisms; however, in general they have a high impact on cell survival and metabolism, affecting microbial contribution to plant growth. New strategies to solve the incompatibility between pesticides and inoculants are needed. The ones that have been proposed to date are still very modest in terms of demand.

Keywords: rhizobia, plant-growth-promoting bacteria, fungicide, insecticide, herbicide, biological nitrogen fixation, inoculation

Introduction

Technologies and agricultural inputs currently applied for food production are essential for large-scale production and are mandatory to feed a population of more than 7 billion people [1]. Years of research and experiments continually performed facing challenges and technological evolution result in inputs in several fields of science. The 1950s was known as the “Green Revolution” period, marked by the intense modernization of agriculture [2, 3]. Products including new machines and synthetic fertilizers, with an emphasis on nitrogen (N) fertilizers, pesticides, seeds of better quality, improvements in the water supply systems, breeding and genetic engineering are examples of technologies developed at that time and that have gained prominence in agriculture [4, 5].

The main positive result of the Green Revolution was the global increase in food production, thus contributing to the reduction of hunger in the world. However, in the following years, the side-effect of this revolution unfolded [6]. The accumulation of pesticides and chemical fertilizers contributes to the pollution of groundwater and cultivated land, soil degradation, and reduction of biodiversity in different ecosystems [5, 7]. Increased deforestation, soil degradation, and emission of polluting gases into the atmosphere have been increasingly observed [8-10]. Despite the undeniable benefits of the Green Revolution, many of the technologies and inputs generated during that period are broadly criticized today. Currently, efforts have been made towards the development of new technologies and inputs focused on more sustainable systems.

Contemporary scientists have pointed out that we are living in a “New Green Revolution” whose main characteristic, and which differs from that experienced in the 1950s, is the development of environmentally-friendly technologies and products [3, 11-13]. Examples of this new concept include the development of products and techniques such as crop rotation, plant genetic engineering for resistance to pests, diseases, and abiotic stresses such as drought, the use of bio-inputs as activators of soil biota, biopesticides and microbial inoculants, also known as biofertilizers in some countries, with the purpose of partially or fully replacing the use of chemical fertilizers, favoring the growth of plants [14-17].

Although the current movement towards agricultural sustainability has force worldwide, the use of agrochemicals is and will continue to be the reality of most farmers [18]. As such, the common scenario towards improving agricultural sustainability with feasible yields to guarantee food security includes the increasing use of bioproducts, such as microbial inoculants, together with pesticides, which are still indispensable for controlling pests and diseases. Therefore, the compatibility between inoculants and pesticides must be understood.

In general, pesticides contain molecules that are potentially toxic to living cells. Depending on the specificity, pesticides can cause toxicity to cells of microorganisms, animals, and plants, often resulting in death after contact with the product. In agriculture, they are commonly applied to the seeds, soil, and leaves of plants to prevent or control pests and diseases [19]. Usually, pesticides and inoculants are added together on the seeds. Thus, it is necessary to verify whether microbial cells in the inoculants are affected by pesticides, impairing the benefits of inoculation.

We should also mention that anticipated inoculation or pre-inoculation, i.e., treating seeds with pesticides and inoculants several days before sowing, has become increasingly adopted by farmers [20]. However, the microorganisms are subjected to long-term exposure to pesticides, increasing the pernicious effect on the bacteria and resulting, for example, in decreased nodulation in legumes [21-23], lower N accumulation in grains [24, 25], and negatively impacting root development of grasses [26]. These losses may be due to microbial cell death caused by pesticides, as demonstrated in some studies [25-28], in which the longer the contact between bacteria and pesticides, the greater is mortality. Besides, changes in cell metabolism, such as formation of smaller colonies and decreased nitrogenase activity, have been reported [25].

The world will need more food, and to meet this increased demand, there is no doubt that pesticides should be employed. However, there is also an increasing demand for environmentally-friendly inputs, including inoculants, to replace chemical fertilizers. One major challenge is to make the chemicals and biologicals compatible. In this review, we gathered information on the use of pesticides and inoculants, starting with the history, current situation, and results of studies that investigated the effects of fungicides, insecticides, and herbicides on microbial inoculants.

The use of pesticides

Studying planting and cultivation practices in ancient times, historians have reported that civilizations were already searching for effective approaches to protect and preserve their food. For millennia, methods such as burning sulfur, using arsenic, growing toxic species together with the crop of interest, and using salts and ashes against weeds were used to protect crops [29, 30]. In one of the oldest documents, from approximately 1550 BC, called Ebers Papyrus, interesting information about techniques used to eliminate insects from food planting areas has been described [30]. The report describes a mix of mercury and arsenic that was used for pest control [30], and a century later, arsenic was used along with honey, especially against ants. In 1867, during a potato beetle [*Leptinotarsa decemlineata (Say*)] outbreak in Colorado, USA, arsenic was used for pest control [29]. During the 1850s, a vineyard owner from Bordeaux, France, applied a mixture of copper and lime to grapes. Initially, the objective was to keep thieves away from his vineyards, but the wine producer realized that the application resulted in lower incidence of diseases. Notably, this mixture is still used today as a fungicide. Over the years, insecticides derived from plants have been discovered, such as pyrethrins and nicotine, the latter used specially to control aphids [19].

In 1939, Paul Müller discovered dichlorodiphenyltrichloroethane (DDT), which was the first modern pesticide. This product played an important role during World War II when it was broadly used to control diseases transmitted by insects, such as malaria and typhus, ensuring soldiers´ health. The discovery of DDT resulted in considerable benefits to agriculture and human health, resulting in Müller being awarded the Nobel Prize in Medicine in 1948. Numerous other cheap and effective synthetic organic pesticides have been developed, contributing to a breakthrough in the market and starting a new era in pest and disease control [19, 29-31]. Fungicides such as captan and glyodin, the insecticide malathion, and the herbicide triazine were introduced in the following decades [29].

The use of pesticides increased until 1962 when the development of new products began to slow down because of the first studies and reports on the environmental and health risks associated with the indiscriminate use of pesticides. The book “Silent Spring” (1962), authored by the American scientist Rachel Carson, played an important role in the history of pesticides. In the book, the author discusses the harmful effects caused by the field spraying of several pesticides containing chlorinated hydrocarbons, among them the most important at the time, DDT. The effectiveness of these products is closely related to their stability and persistence in the environment, but they are also able to accumulate in the adipose tissue of some animals, a process known as bioaccumulation, which in some cases results in biomagnification, factors that make these compounds highly dangerous [19, 29, 30, 32]. Another important finding was the confirmation of the housefly (*Musca domestica*, L). resistance to DDT in Sweden only after a few years of application, another negative point for the use of this product [19].

As a result, in 1972, the US Environmental Protection Agency (EPA) banned the use of DDT in the country, and several pesticides were classified as restricted use, for example, endosulfan, dieldrin, and lindane. Organophosphorus and carbamates, which have lower risk, had been suggested as alternatives [19, 29]. DDT was banned in several other countries and, in

2001; during the Stockholm Convention, 179 nations signed a treaty that agreed to ban 12 persistent organic pollutants (POPs). It is interesting to note that since the 1960s, when both pesticides production and use became strictly regulated, alternative methods for pest and disease control began to be studied, with an emphasis on biological control (BC) [19, 29, 30] and integrated pest management (IPM) [33]. The principle of IPM is based on understanding population dynamics and using actions compatible with the environment to minimize the incidence of pests. In 1998, Kogan defined IPM as “the intelligent choice and use of control tactics that will produce favorable consequences from an economic, ecological, and sociological point of view.” In conclusion, the principle is to use several compatible techniques to keep the pest population at levels below capacity of causing economic, social, and environmental damages. IPM is currently widely used in crops around the world and is responsible for excellent results like reduced use of chemicals and increase in yields, in addition to other traditional methods such as mechanical and physical control and use of resistant plants, but they are not able to fully replace the use of chemical pesticides, which are still used on a large scale [34].

In 1990, the average worldwide use of pesticides by cultivation area was OF 1.5 kg ha^{-1}. Almost 30 years later, this value grew considerably, reaching an average of 2.63 kg ha^{-1} in 2018. The continents that applied more pesticides in 2018 were Asia and America, reaching 3.67 kg ha^{-1} and 3.52 kg ha^{-1}, respectively. Europe applied 1.66 kg ha^{-1} of pesticides, while in Africa the average application was 0.3 kg ha^{-1} [18]. Among Asian countries, China, Japan, and Korea had the highest averages of pesticides per hectare in 2018, reaching 13.07 kg ha^{-1}, 11.84 kg ha^{-1}, and 11.73 kg ha^{-1} respectively. High rates were also reported in 2018 for South American countries, including Ecuador (25.8 kg ha^{-1}), Uruguay (8.16 kg ha^{-1}), Brazil (5.94 kg ha^{-1}), Chile (5.86 kg ha^{-1}), and Argentina (4.29 kg ha^{-1}), while the United States of America (USA) applied an average of 2.54 kg ha^{-1} of pesticides in the same year [18].

Each country has its own laws and regulations regarding the production, commercialization, and use of pesticides, designed mainly to protect human and environmental health. Among the actions of regulatory agencies are, for example, limitation of species to which a certain pesticide can be used, the requirement to use protective equipment, and the total prohibition of any product that is proven to be dangerous, which cannot be reliably mitigated [35]. Estimates point that since 1970, 508 types of active ingredients have been used in the USA, 134 of which were banned, with the majority of the cancelations taking place voluntarily by the manufacturers; only 37 prohibitions came from judiciary decisions. The United States are behind when banning pesticides, probably due to deficiencies in the legislation. From the list of pesticides still in use in the United States, 72 of them have been banned in the European Union, 17 in Brazil, and 11 in China [35].

Several studies have been carried out in the past few decades to understand the damage to human health and environment caused by of certain chemical pesticides [36-38]. These studies are very important because they generate information to guarantee food security. However, crop management still requires the application of pesticides to achieve high yields to meet the world´s increasing demand for food. Given this scenario, the indications are the use of pesticides, but with a trend toward more environmentally-friendly formulations as the replacement by biological control.

Inoculants or biofertilizers

Following the trend of agricultural production and concern about environmental sustainability, an innovative biotechnological product based on living microorganisms capable of making nitrogen available to plants was patented in 1896 and launched in 1898 by the first inoculant producing company, Nitragin; thus, replacing the application of potentially polluting N fertilizers. The first inoculant contained nitrogen-fixing rhizobia for soybean [*Glycine max*

(L.) Merr.] crop [17, 39-41]. Rhizobia are diazotrophic bacteria with an enzymatic apparatus to realize the biological nitrogen fixation (BNF) process, in which atmospheric nitrogen (N_2) is converted into ammonia (NH_3) and further to organic compounds that are easily assimilated by plants (Fig. 1). Therefore, when diazotrophic microorganisms are associated with specific plants, they supply nitrogen to their host, contributing to their development, and in return receive photosynthates from the host plant for their metabolism [42, 43].

Since the first inoculant was launched, a variety of inoculants has been produced, including rhizobia and other plant growth-promoting bacteria (PGPB) [17]. It has been necessary to research on several fronts, including the selection of microorganisms for each plant species [44-49], development of the culture media [50, 51], search for inoculant vehicles [52-55], development of large-scale production, product distribution logistics, methods of inoculation [56-58], among others._Such studies have been responsible for expanding, diversifying, and improving the quality of inoculants. There are several reports on the contribution of inoculants increasing yields of crops at a low cost and mitigating environmental impact [15, 17].

As the production and commercialization of inoculants have increased, some countries have created laws to standardize, supervise, and guarantee the safety and quality of these bioproducts. In 1954, a microbiology professor at the University of Sydney, Australia, listed basic recommendations for quality control and use of legume inoculants, establishing the first quality control laboratory in the country. The Australian Inoculant Research Group (AIRG) is responsible for quality control. Since 2010, Australian inoculants that meet quality standards have exhibited a trademark called “Green Tick Logo,” which certifies that the product contains the correct strain, the number of living cells equal to or above the standard, and the minimum number of contaminating organisms [59]. Australian legislation served as a basis for legislation in other countries, such as in Uruguay and Brazil. In Brazil, legislation was established in 2004 and updated in 2011 [60-62]. Australia has moved to voluntary participation in the quality control of commercial inoculants, while in other countries such as France and Canada, as well as in many South American countries, participation is mandatory. The third group, including the US, comprises only internal control by the industry [17, 63].

Brazilian standards include a list of bacterial strains authorized for inoculation in different plant species, establishing a minimum cell concentration, and the limits for contaminants. Inoculants containing rhizobial species must present 1×10^9 viable cells per gram or mL until the expiration date, which must be at least 6 months, but other species may register with lower concentrations [61, 62]. The maintenance of cell concentration contributes for achieving the desired performance and ensuring product quality, but it is a challenge for the industry, as several factors can affect cell survival such as temperature, pH, drought, light, and availability of nutrients [50, 51, 53, 63, 64].

Soybean inoculation is certainly the most successful example worldwide, with an emphasis in South America. For example, in Brazil, it is well known that inoculation with elite *Bradyrhizobium* spp. strains fully supplies the plant´s demand for nitrogen, avoiding the use of N-fertilizers even with high-yielding genotypes [15, 42]. In the 2019/20 crop season, approximately 36 million hectares were cropped with soybean in Brazil, and even though most of the area had been inoculated in previous seasons, about 80% of the farmers adopted annual inoculation [65, 66]. Traditionally, soybean in Brazil has been inoculated only with *Bradyrhizobium* spp. strains [15]; however, in the past five years, co-inoculation of *Bradyrhizobium* spp. with the PGPB *Azospirillum brasilense* has been increasingly used, so that in a short period it has been adopted by 25% of the farmers [65, 66]. The main trait of the *A. brasilense* strains used for co-inoculation of soybean in Brazil has been recognized as the synthesis of phytohormones [67, 68].

Besides phytohormones synthesis, beneficial properties associated with PGPB include

BNF, phosphate and potassium solubilization, production of siderophores, detoxification of heavy metals, induction of plant systemic tolerance to abiotic and biotic stresses, production of hydrolytic enzymes, and production of exopolysaccharides [69-75] (Fig. 1). Such properties have been reported in several microorganisms, and the most commonly cited carrying one or more of these properties are *Azospirillum* spp. [72, 76], *Pseudomonas* spp. [77-79], *and Bacillus* spp. [80, 81], among others [71, 82]. With such a range of important properties and taking advantage of different microbial processes, the inoculation with mixes of bacteria has gained increased attention [67, 68, 82-84]. Some proposed mixes combine several species [75, 82], but one knows that it is difficult to grow and maintain proper concentrations of bacteria with different metabolic needs, In addition, a thorough preliminary study must be carried out so that the chosen species are compatible with each other.

Before being used as a co-inoculant for legumes, PGPB such as *A. brasilense* has long been used in the inoculation of non-legumes, especially grasses such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), and rice (*Oryza sativa*). Inoculants containing *A. brasilense* have been commercialized for more than 20 years; since 1996 in Argentina [76], 2002 in Mexico [85], and 2009 in Brazil [17, 44, 66]. *A. brasilense* can fix nitrogen, but in much lower quantities than rhizobia, making necessary supplemental N-fertilizer. Possibility of reduction of approximately 25% of the N-fertilized has been observed when *A. brasilense* is used as an inoculant in maize and this is environmentally and economically important [44, 57, 66, 86]. One of the greatest benefits of *A. brasilense* is its ability to produce phytohormones, such as auxins, that stimulate root development [72, 87] and gibberellins [87]. In addition, *A. brasilense* can induce plant defense mechanisms under abiotic and biotic stresses [69, 70, 72, 73]. Due to excellent results of inoculation with *A. brasilense* in several crops [88], it is expected that this practice will continue to grow in the coming years. In Brazil, in a short period of 10 years, since the first commercial inoculant containing *A. brasilense* was launched, the number of commercialized doses reached 10.5 million in 2020 [66].

Rhizobial inoculants have been traditionally used in pasture-growing legumes, mainly alfalfa (*Medica sativa* L.) [89]. However, as most pastures worldwide have grasses, the use of other PGPB in important pastures such as brachiarias (*Urochloa* spp.) have increasingly attracted attention, with results showing improvement in soil fertility, biomass yield, and nutrient content in the forages (Fig. 1) [12, 90-92]. The inoculation pastures of grasses with PGPB has environmental and economic importance because most of the pastures worldwide are at some stage of degradation [93, 94]. By offering better quality fodder to cattle, the pastures can hold a larger number of animals, making saving of other areas from transformation into pastures.

There are different ways to deliver the inoculants to the crops. Seed inoculation is the most common and easy method, as it can be carried out using solid or liquid inoculants. In this process, the inoculant is applied to the surface of the seeds, with or without stickers, which are then shaken so that the product spreads evenly. Other methods of applying liquid inoculants are possible, such as spraying on the soil surface or applying the product in the sowing furrow. Another alternative is leaf spraying, which can be done during certain periods of plant growth. Regardless of the strategy of inoculation, it is extremely important to apply the correct dose of the product, according to the manufacturer's recommendations, as lower or higher cell concentrations may decrease inoculation efficiency [56, 57, 67, 95].

Evidences of Incompatibility between Pesticides and Inoculants

As the benefits of inoculation are closely related to the establishment of a plant-microorganism interaction, the survival and maintenance of microbial properties are crucial and mandatory. Therefore, the evaluation of microbial survival at the time of inoculation, and of the effectiveness on the inoculated crop are critical. The most common situation in commercial crops is the combination of several products for different purposes, such as soybean seed treatment with microbial inoculants for nitrogen fixation, fertilizers and pesticides for prevention or treatment of pests and diseases. In many cases, depending on the mode of application, one product ends up being exposed to the other, or interacting one another either on the seeds, propagated material, in the soil, or leaf surface. It is important to know whether the contact of pesticides with microorganisms in the inoculant can affect cell survival and metabolism. Concerns about the compatibility of agrochemicals with microbial inoculants have been raised for decades, and several studies have shown that the impact of chemicals on the inoculant depends on the active ingredient, presence of other chemical substances that make up the formulation of pesticides, mechanism of action (systemic or contact), and method of application. The effects of incompatibility also depend on the bacterial species present in the inoculant, which may have different responses. However, few species have been evaluated for this purpose. The most recurring ones belong to the genres *Rhizobium* spp., *Bradyrhizobium* spp. and *Azospirillum* sp.

Compatibility with Fungicides

Fungicides are chemical products formulated to prevent the infection of plant tissues by phytopathogenic fungi, and in some cases, capable of extending the control of diseases caused by bacteria and viruses. The control exerted by fungicides can be mediated by killing the pathogen, temporarily inhibiting its germination and growth, or by affecting the production of spores [96]. Over the years, several fungicides have made it to the market; some have stood out and remained at the top in the list of the most used fungicides until today, more effective products have replaced others, and some have been banned. Fungicides of contact generally do not have a specific mode of action, are highly toxic, and when applied to seeds, soil, and plant leaves limit the pathogen survival. The most common are thiram (dithiocarbamate), captan (quinone and heterocyclic), exon (aromatic), and guazatine. Upon entering microbial cells, the molecules promote a series of chemical reactions in nucleic acids and their precursors, and metabolic routes, affecting cell survival [96].

The majority of studies on compatibility have been performed with fungicides and microbial inoculants carrying rhizobia. Fungicides may affect several steps of the symbiosis, from the survival of the rhizobia on the seed to nodule formation and N_2 fixation efficiency; in general, studies have been performed with soybean (e.g., 53, 97-99). The use of pesticides intensified in the past two decades, and so did concerns about their compatibility with inoculants [17].

Brikol et al. [21] evaluated the effects of applying different concentrations of the fungicide Thiram (10 to 750 µg mL^{-1}) on soybean seeds inoculated with *B. japonicum*, which were grown under greenhouse conditions for 75 days. They observed that concentrations above 100 µg mL^{-1} reduced nodule number and dry weight, as well as the activity of the bacterial enzyme nitrogenase, responsible for the nitrogen fixation process. Similarly, there are reports [24, 100] of decrease in soybean nodulation and N accumulation in plants when seeds were inoculated with *B. elkanii* (SEMIA 5019) and *B. japonicum* (SEMIA 5079) and treated with different fungicides, benomyl, captan, carbendazin, carboxin, difenoconazole, thiabendazole, thiram, and tolylfluanid. Changes in nodule number were also observed in a field trial by Zilli et al. [101] when soybean seeds were treated with either carbendazin + thiram or carboxin + thiram.

Interestingly, results of some studies indicate differences between strains in their tolerance to fungicides. For example, in soybean seeds treated with carbendazim + thiram, the lowest tolerance was observed in *B. elkanii* amongst four soybean *Bradyrhizobium* strains used in commercial inoculants in Brazil [*B. elkanii* (SEMIA 5019 and SEMIA 587), *B. japonicum* (SEMIA 5079), and *B. diazoefficiens* (SEMIA 5080)] [101]. In another study, Gomes et al.

[102] observed no effects on nodulation when seeds were inoculated with *B. japonicum* SEMIA 5079 + *B. diazoefficiens* SEMIA 5080 and treated with carbendazin + thiram. More recently, when the compatibility of *B. japonicum* SEMIA 5079 and *B. elkanii* SEMIA 587 was verified with Standak®Top, composed of a mixture of two fungicides and one insecticide (piraclostrobin, thiophanatemethyl, and fipronil), the effects were also more drastic for *B. elkanii* [25]. Altogether, indications are that *B. elkanii* is less tolerant to fungicides than *B. japonicum* or *B. diazoefficiens*.

It is reasonable to postulate that the main effects of the fungicides used in seed treatment would be the decrease of rhizobial survival or inhibition of the root infection process, consequently affecting nodulation and BNF, and as a result grain yield, as observed by Zilli et al. [101]. In that study, the grain yield reduced by 20%, in addition to a decrease in the N content in grains when seeds were treated with *B. elkanii* SEMIA 587 + carbendazin + thiram (Fig. 2). However, some reports have indicated that the effects of fungicides may appear later. For example, in a study by Gomes et al. [102], although fungicides (carbendazin + thiram) did not affect nodulation, plants had lower number of pods per plant, grains per plant, and yield. Intriguingly, in two field experiments performed with soybean in Brazil, seed treatment with Standak®Top affected the total N accumulated in the grains of plants relying on both BNF and N fertilizer, indicating the negative impact of the pesticide on N metabolism [25]. In another study, a decrease in both protein and oleic acid contents was observed in soybean inoculated and treated with mefenoxan + fludioxonil [103].

As mentioned previously, negative effects may be related to the toxicity of fungicides on microbial cells, followed by impacts on microbial metabolism, reducing the effectiveness of the inoculant. Ahmed et al. [104] evaluated the growth of *Bradyrhizobium* sp. and *Rhizobium* sp. in Petri dishes containing solid culture medium and different concentrations of fungicides (captan, thiram, luxan, milcurb, and frernasan-D), soaked in discs placed on the medium. The bacteria were tolerant to concentrations below 100 µg L^{-1}, but at 1000 µg L^{-1} inhibition of growth and a decrease in the colony diameter of the surviving cells were observed (Fig. 2). Rathjen et al. [105] also reported that *Rhizobium leguminosarum* bv. viciae (WSM1455) was sensitive to the fungicides Thiram 600 and P-Pickel T (PPT), and their active ingredients (thiram and thiabendazole) at concentrations above 200 µg $disc^{-1}$, with growth halos greater than 10 mm around the disks containing the fungicide.

To verify the survival of *B. elkanii* (SEMIA 5019) and *B. japonicum* (SEMIA 5079) on seeds treated with fungicides, Campo et al. [24] recovered and counted viable cells from seeds inoculated and treated with benomyl, captan, carbendazin, carboxin, difenoconazole, thiabendazole, thiram, or tolylfluanid. After only 2 h of contact with carbendazin + thiram, viable bacterial cells were reduced by up to 64%, and reached 83% after 24 h. Mortality was verified with all other fungicides, reaching 95% with the mixture of thiabendazole and tolylfluanid after 24 h of contact. In addition to rhizobia, fungicides can also impair the contribution of other PGPB. The toxic effect of the combination of carbendazin + thiram was also verified for *A. brasilense* strains Ab-V5 and Ab-V6 by Santos et al. [27]. In that study, a decrease in the recovery of viable cells from seeds only after 2 h of contact was observed, and the viable cell count drastically decreased after 24 h, in comparison with the seeds not treated with fungicides (Fig. 2).

Compatibility with Insecticides

Many herbivorous insects feed on plants during their larval and adult stages, and/or some are important vectors of plant diseases. In both cases, insects may cause serious damages to crops. Insecticides, usually synthetic chemicals, acting as ovicidal, larvicidal, and adulticidal agents are used to prevent growth or kill insects [106]. Neonicotinoids, organophosphates, diamides, pyrethroids, and carbamates act on nerves and muscles; phosphides, cyanides, and carboxamides on respiration, and cyclic ketoenols and ecdysone are agonists that interfere with insect growth and development [107].

Rathjen et al. [105] evaluated the *in vitro* toxicity of an imidacloprid-based insecticide on *R. leguminosarum* bv. viciae (WSM1455), and *Mesorhizobium ciceri* (CC1192). The strains were applied on solid culture medium in Petri dishes and sterile filter discs containing different concentrations (0, 100, 200, and 300 µg discs^{-1}) of the insecticide. The inhibition of *R. leguminosarum* growth was observed at all concentrations. In alfalfa (*Medicago sativa* L.), Fox et al. [22] reported that seed treatment with the insecticides methyl parathion, DDT and pentachlorophenol affected the symbiosis with *Ensifer* (syn. *Sinorhizobium*) *meliloti*. The insecticides not only reduced nodule number and dry weight but also nitrogenase activity in nodules and plant biomass production. Ahemad [23] also reported several negative effects with the application of pyriproxifene, at the recommended dose of 1,300 µg kg^{-1} soil, in chickpeas (*Cicer arietinum* L.), peas (*Pisum sativum* L.), mung beans (*Vigna radiata* L. Wiclzek), and lentils (*Lens esculentus, = Lens culinaris* Medik) grown in pots that remained in an open field. Although the plants had not been inoculated, inferring that nodulation was related to indigenous rhizobia, pyriproxifer resulted in a 44% decrease in nodule number in peas, 14% in mung beans, and 5% in chickpeas and lentils, resulting in decreases in nodule dry weight compared with the controls not treated with insecticide. There was also a 17% decrease in the concentration of nitrogen in the roots of chickpeas, 15% in peas, 14% in mung beans, and 18% in lentils, and a 5% decrease in the protein contents in grains of chickpeas, 4% in mung beans, 3% in lentils, and 1% in peas, compared with the controls not treated with insecticide.

Insecticides also affect PGPB other than rhizobia. For example, Fernandes et al. [108] studied the effects of five insecticides (imidacloprid, fipronil, fenamethoxam, endosulfan, and carbofuran) indicated for sugarcane (*Saccharum* spp.) on the diazotrophic bacterium *Herbaspirillum seropedicae*. *In vitro* evaluations of cell growth after 33 h indicated that the insecticides that most interfered with bacterial growth were endosulfan and carbofuran. Madhaiyan et al. [109] evaluated the effect of different insecticides (monocrotophos, malathion, chlorpyriphos, diclorvos, lindane and endosulfan) on *Gluconacetobacter diazotrophicus*, another PGPB found in association with sugarcane. Except for malathion, all other insecticides reduced cell concentration, and lindane lysed the cells (Fig. 2). In the same study, nitrogenase activity was fully inhibited by monocrotophos, dichlorvos, and lindane, 83.3% by chlorpyriphos, 80.9% by melation, and 33.4% by endosulfan. Concerning the synthesis of indoleacetic acid (IAA) and gibberellin A3 (GA3) by *G. diazotrophicus*, inhibition was observed with lindane, with decreases of 93.2% and 96.5% for IAA and GA_3, respectively. The authors also described that the insecticides dichlorvos, chlorpyriphos, and lindane completely inhibited the solubilization of phosphate (P) and zinc (Zn) [109] (Fig. 2).

Compatibility with Herbicides

Another class of important pesticides for agriculture are herbicides used for weed control. Herbicides have different degrees of specificity based on differences in biochemical pathways in certain plant groups. The mode of action of herbicides is generally related to cell division process, which inhibits a key enzyme/protein [110]. Examples of herbicides applied worldwide include glyphosate, paraquat, and diuron. Among these, the most well-known is glyphosate; since its introduction in the 1970s, its use spread quickly, facilitated cropping, but also implied in the growing appearance of resistant weeds, resulting from a natural process of plant adaptation, and decreasing its efficacy [111-113]. An important alternative to minimize this problem, in addition to integration with other control methods, would be diversification in the use of herbicides, including others with different mechanisms of action [114].

Few studies have investigated the compatibility between herbicides and inoculants. Madhaiyan et al. [109] evaluated the effects of different herbicides (butachlor, alachlor, atrazine, and 2,4-D) in liquid culture medium on the growth and metabolism of *G. diazotrophicus*. Cell growth was impaired only in the presence of 2,4-D, but all herbicides reduced nitrogenase activity, the production of IAA and GA_3, and the solubilization of P and Zn. The highest inhibition of nitrogenase activity (73.6% and 65.3%) was observed with alachlor and atrazine, respectively, while butachlor mostly affected production of IAA (53.3%), whereas 2.4-D mostly affected the production of GA_3 (78.8%). Butachlor was also responsible for the strongest reduction in the solubilization of P and Zn. Therefore, although herbicides do not affect cell survival, they significantly affect metabolism of *G. diazotrophicus* [109].

In an assay performed under greenhouse conditions, Angelini et al. [115] evaluated the effects of imazetapir, imazapic, S-metachlor, diclosulam, and glyphosate on diazotrophic bacteria in the soil during the cultivation of peanuts (*Arachis hypogaea* L.). The seeds were sown and the herbicide was sprayed on the soil surface. All herbicides caused reduction in cell concentration in both free and symbiotic diazotrophic bacteria, and this negative impact was confirmed under field conditions even one year after the application. Nitrogenase activity also reduced due to herbicides, except for glyphosate [115].

The ability of cyanobacteria to fix atmospheric nitrogen in flooded soils suitable for rice cultivation, make this group an important ally in maintaining soil fertility and contributing for cereal yield. Thus, Dash et al. [116] evaluated the responses of cyanobacteria in rice plantation soil to the exposure of different agrochemicals, including the herbicide benthiocarb that was applied in one dose at the time of puddling. The herbicide decreased cell growth, which was even worse when combined with urea (used as a fertilizer). Benthiocarb reduced nitrogenase activity between 13-27%, compared with the control without herbicide, and its combination with urea resulted in an even greater reduction in addition to a decrease in the nitrogen accumulation that reached 47% at 60 days.

Concerning the symbiosis between legumes and rhizobia, in general, herbicides have been considered less toxic than fungicides and insecticides [100], with glyphosate being the one with lower toxicity [117; 118]. Although the negative effects of glyphosate on *B. japonicum* growth were reported by King et al. [119], the doses in the experiment were far higher than those recommended for field application. With the release of genetically modified (GM) genotypes tolerant of herbicides, studies on the compatibility with the GM genotypes and herbicides have begun. In soybean, which represents the most used herbicide-tolerant species, glyphosate-resistant (Roundup Ready) pairs of nearly isogenic cultivars were evaluated in six field sites in Brazil for three crop seasons. Although the transgenic trait negatively affected some BNF variables, these effects had no significant impact on soybean grain yield, and no consistent differences between glyphosate and conventional herbicide application were observed on BNF-associated parameters [120]. Similar results were reported in 20 field trials performed with soybean with the *ahas* transgene, imidazolinone, and conventional herbicides [121]. Finally, it is worth mentioning the reported capacity of several rhizobia to degrade herbicides, such as glyphosates [122, 123], contributing to a decrease in their negative impact.

Compatibility with mixtures (fungicides, insecticides, and herbicides)

Approximately 70% of the pesticides available in the market contain mixtures of two or more types of fungicides and insecticides [124] and are often combined with herbicides at the time of application, aiming to facilitate the combined control of pests, diseases, and weeds. However, the damage to microbial inoculants increases with the number of combined chemicals. As previously mentioned, Standak® Top, one of the most used for treatments of seeds in several countries, especially for soybean, is composed of two fungicides and one insecticide; it affects the survival of *B. japonicum* and especially *B. elkanii* cells, with a drastic decrease verified after 7 days of contact [25]. It is worth mentioning that pre-inoculated soybean seeds have been in contact with Standak®Top for up to 90 days, often resulting in zero recoveries of rhizobial cells from seeds [124].

Another interesting observation in the study by Rodrigues et al. [25] was the changes in colony morphology, smaller with the increase of the exposure to the pesticide. However, regular colonies were recovered after the bacteria were grown under optimal conditions, indicating an adaptive mechanism to the stressful conditions when exposed to the pesticides.

Santos et al. [26] evaluated the compatibility of Standak®Top with *A. brasilense* strains Ab-V5 and Ab-V6. First, differences were observed between strains, with lower tolerance of Ab-V5, so that after 24 h of exposure the recovery of viable cells dropped from 4.56×10^5 to 4.37×10^2 cells seed^{-1}. In a greenhouse experiment with the combined strains, Standak®Top decreased the number of lateral roots and root hairs and resulted in shorter root hair length.

Pereira et al. [28] also reported the mortality of *A. brasilense* strains Ab-V5 and Ab-V6 in maize seeds treated with a mixture of fungicides and insecticides (metalaxyl-M + fludioxonil + thiamethoxam + abamectin). The cell survival rate after 12 h of inoculation of seeds treated with the pesticide was only 13.56%, whereas in untreated seeds was 65.87%.

Are there alternatives to the challenges of using pesticides and microbial inoculants?

Remarkable advances in genetic engineering have occurred particularly in the last decade and are advancing towards obtaining plant genotypes resistant to abiotic stresses, pests and diseases [125-127]. However, large-scale agriculture to feed the increasingly growing population still demands the heavy use of pesticides for at least two more decades. Practical agriculture without pesticides may be a dream for most of our society but is currently restricted to a few farmers, most in small properties, and there is no technology in the research pipeline to make it feasible for the majority of the cropped areas in a short time. On the other hand, the use of bioproducts aimed at the total or partial replacement of chemicals used for the control of pests, diseases, weeds, and fertilizers is growing at a rate never seen before [17]. The major limitation, as we have shown in this review, is the low compatibility between pesticides and microbial inoculants applied to seeds. There is an urgent need to develop alternatives to make microbial inoculants compatible with pesticides.

Ahemad and Khan [128] selected strains of *R. leguminosarum* that were tolerant to high concentrations of the insecticides fipronil and pyriproxifene used in peas. The tolerant strain MRP1 was characterized as the highest in the synthesis of IAA, siderophores, and EPS; in a field trial performed in soil previously treated with insecticides, the strain contributed to the significant increases in nodulation, N and P contents of roots and shoots, and grain yield. The results show the feasibility of selecting strains tolerant of pesticides. One main limitation is the increasing number of pesticides used in agriculture, which would require multiple steps of microbial selection.

To develop pesticides less harmful to microbial bioinoculants, the inclusion of carriers for the active ingredients might be simpler than obtaining tolerant strains. Unfortunately, the pesticide industry has not demonstrated interest in following this strategy, probably because the chemical industry is financially more powerful than the inoculant industry, and the appeal to invest in less toxic molecules.

Another strategy could be to take advantage of microbial metabolites instead of living microbes [87]; however, this is only applicable to microorganisms that produce secondary metabolites useful to the host plants, like *Azospirillum* sp. in grasses and pastures, and not by mechanisms that require living microorganisms, such as rhizobia, to nodulate legumes.

Investment should be made on innovation in formulations, including cell protection to minimize or avoid the toxic effects of pesticides on microbial cells. The addition of protective molecules such as polymers, chemicals, or synthesized by microorganisms may help in this regard, for example, polyhydroxyburytate (PHB) [50, 129, 130] and biofilms [131].

In Brazil, with no short-term solutions for compatibility in sight, the only feasible strategy is physically avoiding the contact of inoculants with pesticides. Seed treatment with pesticides, for example, for soybeans that currently may contact up to 14 chemical products, an in-furrow application of biologicals has proven to be effective in guaranteeing the benefits to the crop. Despite the need of increasing the dose of inoculant applied in-furrow, the cost of inoculant is low in comparison with the benefits. A pioneering study confirming that the in-furrow inoculation of soybean with 2.5 times the concentration used for seed inoculation alleviated the effects of seed treatment with agrochemicals was published in 2010 [56]. Despite requiring that the farmers buy new equipment, 20% of the farmers in Brazil adopted this technique by 2020. Other strategies, with an emphasis on soil-spray and leaf-spray inoculation [57, 86, 132, 133] have also been investigated and show some degree of effectiveness, but are not as effective as in-furrow and seed inoculations.

Final Remarks

The critical analysis of this review points to some certainties: (*i*) considering the technologies available today and those that should be available in the next few years, large-scale agriculture to meet the increasing food demand will require high inputs of pesticides; (*ii*) the demand for higher sustainability in agriculture, with bioproducts aiming at partially or fully replacing pesticides and fertilizers is increasing; (*iii*) inoculants or biofertilizers have been increasingly adopted by farmers, but their compatibility with pesticides, especially when used for seed treatment, is very low; (*iv*) strategies to solve the incompatibility between pesticides and inoculants are needed, as those proposed until now are still very modest with regards to their feasibility.

Incompatibility between pesticides and inoculants affects cell survival and metabolism. The level of incompatibility with the pesticides depends on the active principle, formulation, doses, time of contact with the cells, and may vary with the bacterial species or strain. Despite the increasing market for biological products aiming at more sustainable agriculture [134], very few intellectual and economic investments have been made to search for compatibility of biological products with chemicals. Therefore, there is an urgent need to emphasize studies and development of innovative strategies to mitigate the incompatibility between pesticides and microbial inoculants.

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Abbreviations

All abbreviations have been cited in their complete forms when mentioned for the first time in the manuscript.

Ethics Approval and consent of participation

The study has not involved any human or animal participation or data

Consent for publication

All authors gave the consent for publication

Availability of Data and Materials

All data and materials cited in the manuscript are freely available for the scientific community.

Competing Interests

Authors declare no competing interests regarding the data or the manuscript

Ethical interests

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Figures Legends

Figure 1 - Main benefits of inoculation with rhizobia and plant-growth-promoting bacteria (PGPB). Biological Nitrogen Fixation (BNF), phosphate (P) and potassium (K) solubilization, phytohormone production (auxins and gibberellins), and induced plant systemic tolerance to abiotic and biotic stresses are represented. As benefits, there are increases in biomass production, yield, and improvement in soil fertility.

Figure 2 - Representation of the effects reported on the incompatibility between pesticides and inoculants, from the moment of contact with microbial cells to damages to plant development.

Table 1 – Effect of pesticides on cell viability and/or morphology and on the metabolism of microorganisms used as inoculants

Pesticide	Concentration	Microorganism	Effect	Reference
Monocrotophos[i], Malathion[i], Chlorpyripho[i], Dichlorvos[i], Lindano[i] e Endosulphan[i]	Recommended dose of each product	*Gluconacetobacter diazotrophicus*	With the exception of Malathion, all insecticides reduced cell viability. Nitrogenase activity was totally inhibited by Monocrotophos, Dichlorvos and Lindano. Production of IAA and GA_3, and solubilization of P and Zn were impaired	[109]
Butachlor[h], Alachlor[h] Atrazine[h] and 2,4-D[h]	Recommended dose of each product		Cell growth was hindered by 2,4-D. All herbicides reduced the activity of nitrogenase, the production of IAA and GA_3, and the solubilization of P and Zn	
Captan[f], Thiram[f], Luxan[f] and Fernasan-D[f]	1000 µg L^{-1}	*Bradyrhizobium* sp. and *Rhizobium* sp.	Decreased colony diameter and inhibited growth in areas close to the fungicide application site	[104]
Benomyl[f], Captan[f], Carbendazin[f], Carboxin[f], Difenoconazole[f], Thiabendazole[f], Thiram[f], Tolylfluanid[f]	Recommended dose for soybean	*B. elkanii* and *B. japonicum*	All fungicides caused mortality of microorganisms	[24]
Imidacloprid[i], Fipronil[i], Thiamethoxam[i], Endosulphan[i] e Carbofuran[i]	250 g ha^{-1}, 400 g ha^{-1}, 480 g ha^{-1}, 2.800 g ha^{-1}, 1.650 g ha^{-1}, respectively	*Herbaspirillum seropedicae*	Endosulphan increased the lag phase. Carbofuran increased generation time and reduced lag phase	[108]
Pyraclostrobin[f], thiophanato-methyl[f] e fipronil[i]	2 mL kg^{-1} maize seed	*A. brasilense* (Ab-V5 and Ab-V6)	Drastic reduction in cell concentration 24 h after inoculation in treated seeds	[26]
Carbendazin[f]+Thiram[f]	40-60 mL 20		Drastic reduction in cell concentration 24 h after	[27]

	kg^{-1} maize seed		inoculation in treated seeds	
metalaxil-m + fludioxonil + tiametoxame + abamectin	Recommended dose for maize		Drastic reduction in cell concentration 12 h after inoculation in treated seeds	[28]
Thiram[f], **Thiram+Thiabendazole**[f] **and PPT**[f]	> 200 µg L^{-1}	*Rhizobium leguminosarum* bv. viciae	Formation of growth inhibition halos greater than 10 mm around the fungicide	[105]
Imidacloprid[i]	0, 100, 200 e 300 µg L^{-1}		Formation of growth inhibition halos greater than 10 mm around the insecticide for all concentrations evaluated	
Pyraclostrobin[f], **thiophanato-methyl**[f] **e fipronil**[i]	Recommended dose for soybean	*B. elkanii* and *B. japonicum*	Drastic decrease in cell concentration after 7 days of exposure. Colony formation with smaller diameter	[25]

[f]fungicide; [i]inseticide, [h]herbicide

Table 2 – Effect of pesticides on the development of plants whose seeds had been inoculated.

Culture	Fungicide	Microorganism	local	Effect	Reference
Soybean (*Glycine max*)	Thiram[f]	*B. japonicum*	Greenhouse	Lower nodule number, nodule dry weight and nitrogenase activity	[21]
	Benomyl[f], Captan[f], Carbendazin[f], Carboxin[f], Difenoconazole[f], Thiabendazole[f], Thiram, Tolylfluanid[f]	*B. elkanii* (SEMIA 5019) + *B. japonicum* (SEMIA 5079)	Greenhouse and field	Reduction in the number of nodules and in the total N in grains	[24]
	Carbendazin[f]+Thiram[f]; Carboxin[f]+Thiram[f]	*B. elkanii* (STRAIN 5019 and STRAIN 587), *B. japonicum* (STRAIN 5079 and STRAIN 5080)	Field	Reduction of nodulation efficiency. Reduction of N content and grain yield to SEMIA 587 with Carbendazin + Thiram	[101]
	Carbendazin[f]+Thiram[f]	*B. japonicum* (STRAIN 5079 and STRAIN 5080)	Field	Reduction in the number of pods per plant and grains per plant	[102]
	Mefenoxam[f]+Fludioxonil[f]	*B. japonicum*	Field	Reduced grain yield and protein and oleic acid content	[103]
	Pyraclostrobin[f], thiophanato-methyl[f] and fipronil[i]	*B. japonicum* (STRAIN 5079) and *B. diazoefficiens* (STRAIN 5080)	Field	Less N accumulation in grains	[25]
Alfafa (*Medicago sativa)*	Methyl parathion[i], DDT[i] e pentachlorophenol[i]	*Sinorhizobium meliloti*,	Greenhouse	Reduction of nitrogenase activity, number of nodules and plant dry weight	[22]
Peanut (*Arachis hypogaea).*	Imazetapir[h], Imazapic[h], S-metachloro[h],	Diazotrophic bacteria present in	Greenhouse	Reduction of cell concentration of free and symbiotic diazotrophic	[115]

	Dichlosulam^{h} and Glyphosate^{h}	the soil		bacteria	
			Field	Reduced cell concentration of free and symbiotic diazotrophic bacteria and reduced nitrogenase activity except for glyphosate	
Chickpeas (*Cicer arietinum* L.), pea (*Pisum sativum* L.), Mung beans (*Vigna radiata* L. Wiclzek) and lentil (*Lens esculentus*, = *Lens culinaris* Medik).	Pyriproxyfen^{i}	Bacteria commonly present in the soil used	Pots in the field	Reduction in the number of nodules, in the dry weight of nodules, in the concentration of N in roots and in protein concentration in the grains	[23]
Rice (*Oryza sativa*)	Benthicarb^{h}	Cyanobacteria naturally found in the rice paddy soil	Field	Decreased cell growth, nitrogenase activity and Naccumulation	[116]
Maize (*Zea mays*)	Pyraclostrobin^{f}, thyophanato-methyl^{f} and fipronil^{i}	*A. brasilense* (Ab-V5 and Ab-V6)	Greenhouse	Fewer branch roots, root hair and shorter root hair length	[26]

^{f}fungicide; ^{i}inseticide, ^{h}herbicide

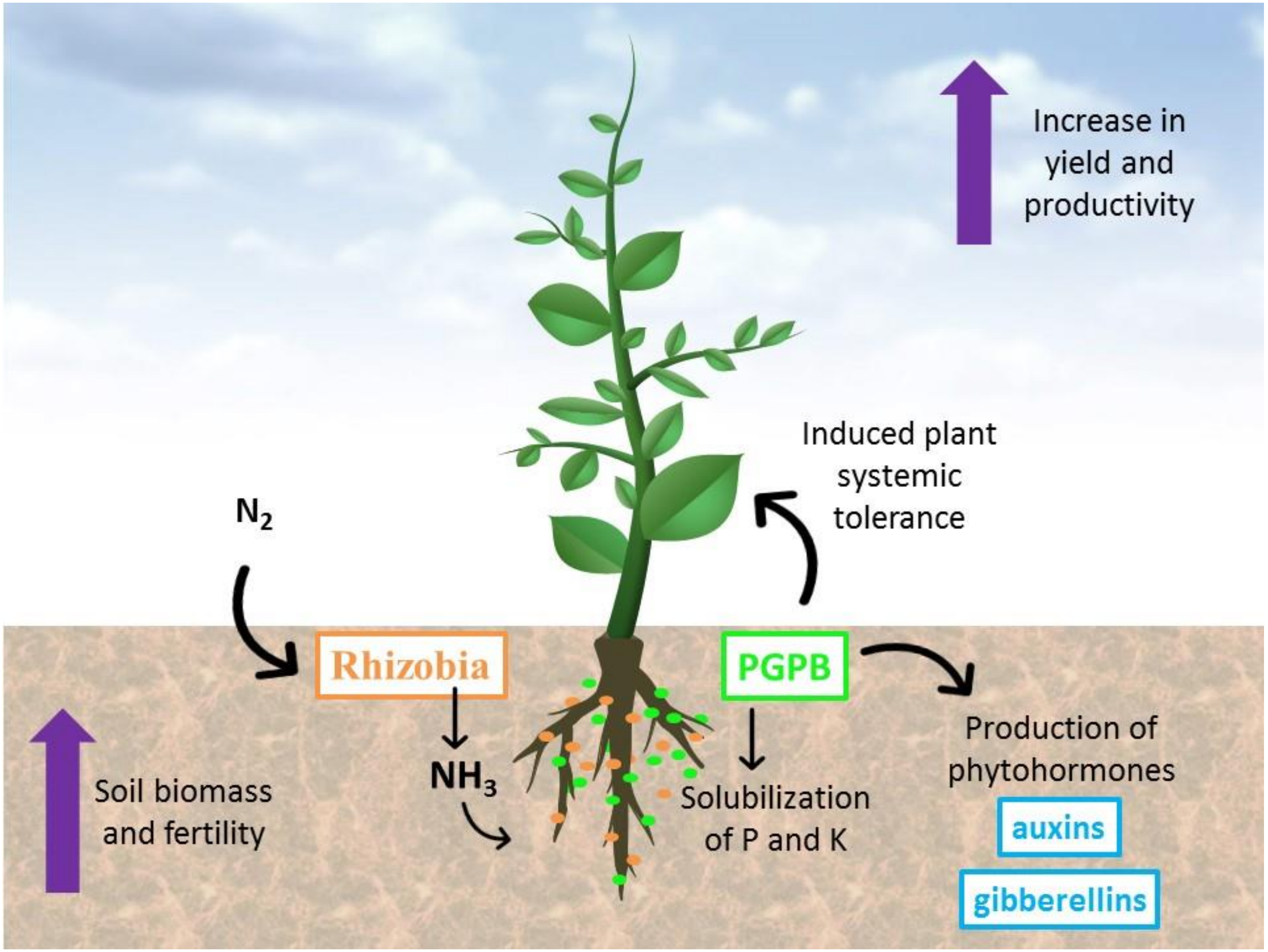
Increase in yield and productivity
Induced plant systemic tolerance
N_2
Rhizobia
PGPB
NH_3
Solubilization of P and K
Production of phytohormones
auxins
gibberellins
Soil biomass and fertility

Fig. 1.

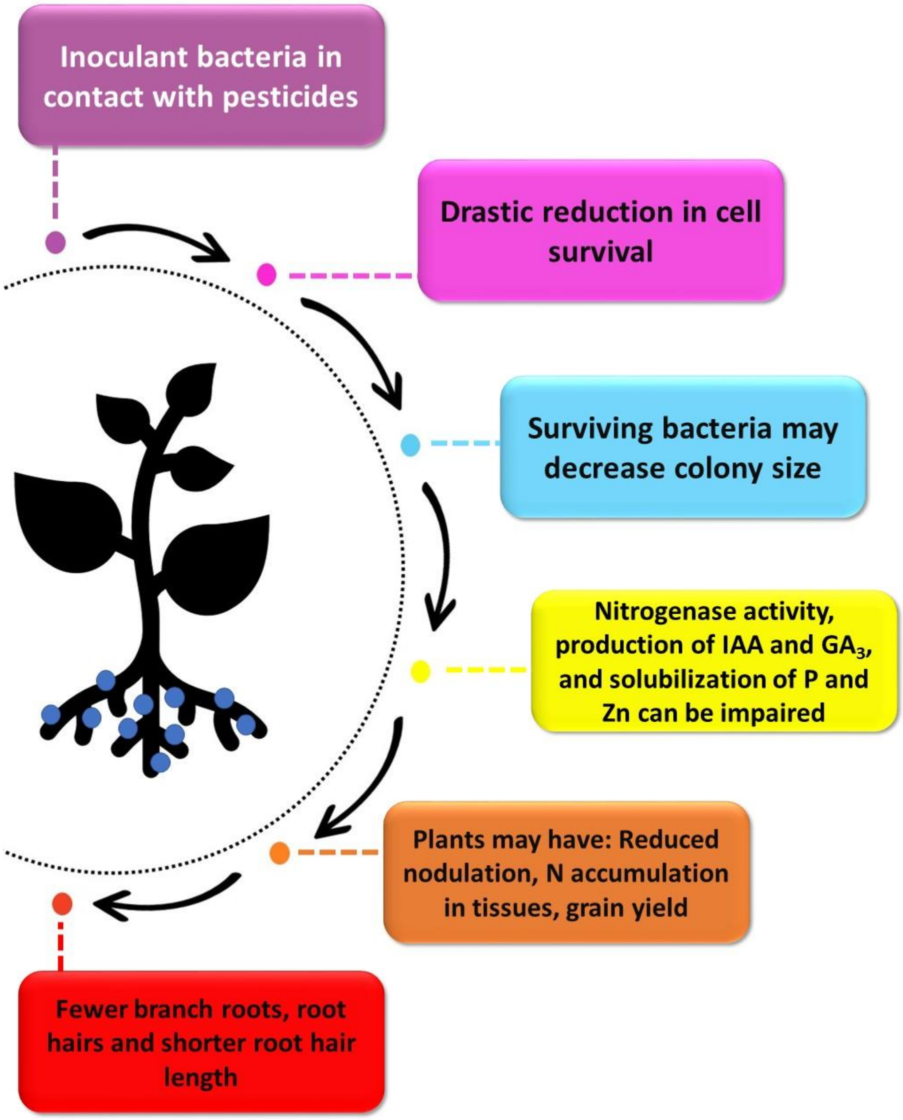
Inoculant bacteria in contact with pesticides
Drastic reduction in cell survival
Surviving bacteria may decrease colony size
Nitrogenase activity, production of IAA and GA_3, and solubilization of P and Zn can be impaired
Plants may have: Reduced nodulation, N accumulation in tissues, grain yield
Fewer branch roots, root hairs and shorter root hair length

Fig. 2

CONCLUSÃO

Com base nos resultados obtidos nos trabalhos apresentados, pode-se concluir que a inoculação de culturas é uma prática capaz de gerar aumento significativo de produtividade. O Brasil é destaque nas pesquisas relacionadas a inoculantes, portanto espera-se que cada vez mais agricultores comecem a aderir e que novas formulações, ainda mais eficientes, cheguem ao mercado.

Apesar dos benefícios resultantes do uso dos inoculantes, quando associados a agrotóxicos pode-se observar que a viabilidade celular de *Azospirilum brasilense* inoculado em sementes de milho cai drasticamente em 24 horas quando as sementes também recebem tratamento com agrotóxicos. Com o desenvolvimento da metodologia para recuperação de células inoculadas em sementes de milho, mais trabalhos poderão ser realizados a fim de se compreender mais detalhadamente esse processo, para diferentes ingredientes ativos usados no tratamento de sementes dessa cultura. Além disso, os benefícios da inoculação do milho com as estirpes Ab-V5 e Ab-V6 podem ser prejudicados, principalmente quanto ao número de ramificações e pelos radiculares, apesar de não parecer interferir no desenvolvimento inicial da planta. Estudos futuros são necessários para observar se há interferência na produtividade do milho.

Levando em consideração que o uso de pesticidas ainda é a forma mais comum para se combater pragas e doenças nas lavouras, torna-se evidente a necessidade de que novas formulações de inoculantes sejam desenvolvidas levando em consideração a vulnerabilidade das células bacterianas em contato com esses produtos químicos, visando a proteção dessas células, a fim de que todo potencial biotecnológico dos inoculantes seja explorado.