Revista Brasileira de Ciência do Solo

Division - Soil Processes and Properties | Commission – Soil Biology

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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Received: July 28, 2020

Approved: November 27, 2020

How to cite: Santos MS, Nogueira MA, Hungria M. Outstanding impact of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 on the Brazilian agriculture: Lessons that farmers are receptive to adopt new microbial inoculants. Rev Bras Cienc Solo. 2021;45:e0200128. https://doi.org/10.36783/18069657rbcs20200128

Editors: José Miguel Reichert, Sidney Stürmer, and Jerri Edson Zilli.

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.

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.

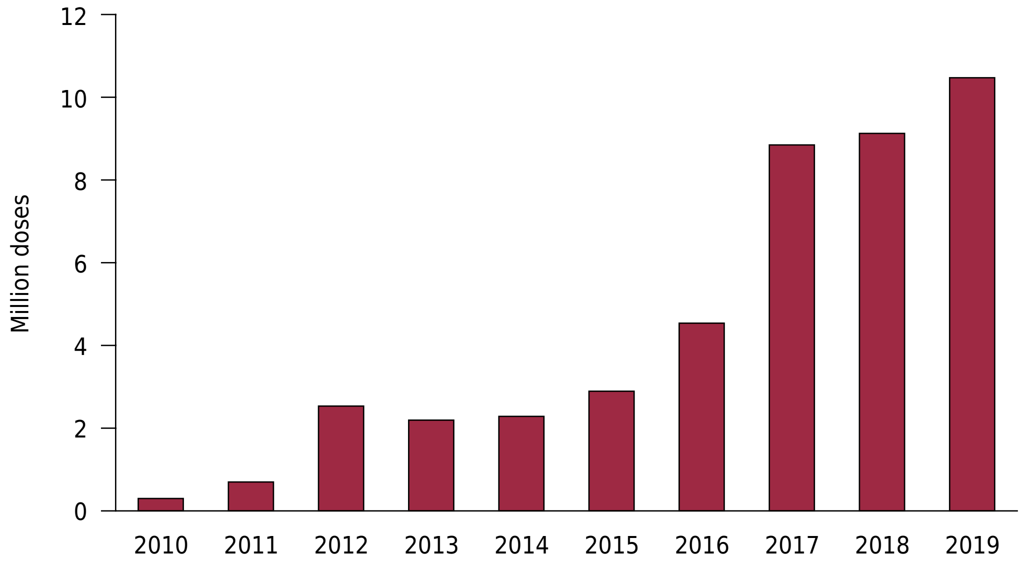

Figure 1. Doses of inoculants carrying *Azospirillum brasilense* strains Ab-V5 and Ab-V6 since the launch of the first commercial inoculant. Numbers based on data from Anpii (2020) and personal communication from the private sector.

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

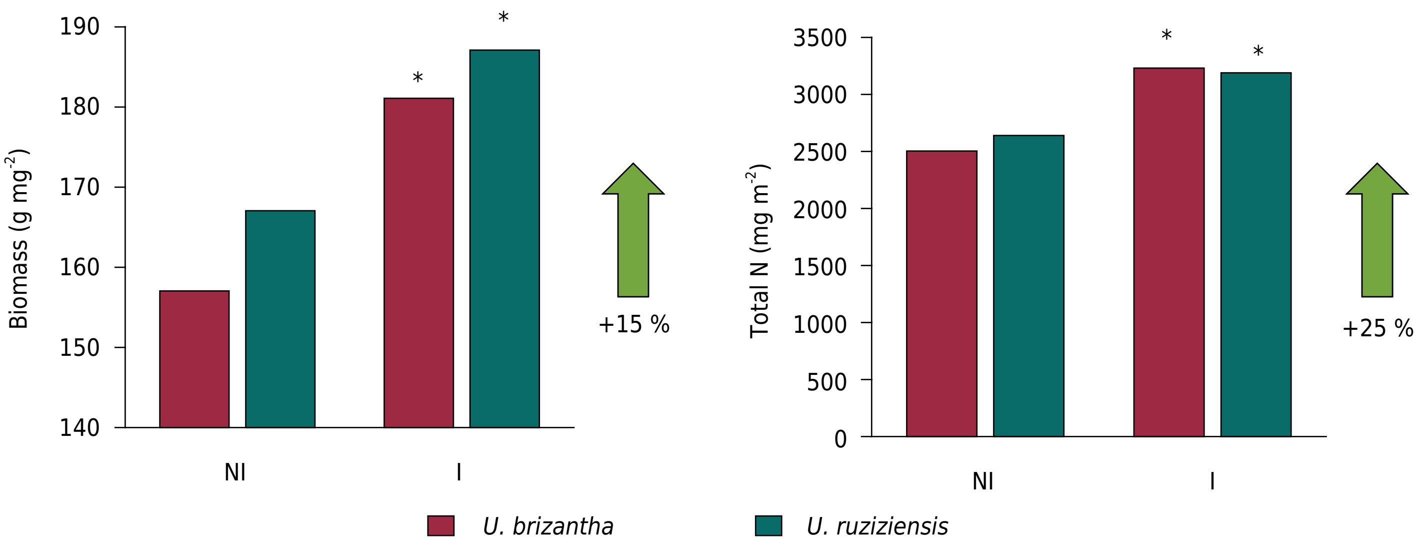

Figure 3. Shoot biomass production and total N accumulated in the biomass of *Urochloa* (=*Brachiaria*) *brizantha* and *Urochloa ruziziensis* inoculated or not with *Azospirillum brasilense* strains Ab-V5 and Ab-V6; all treatments received 40 kg of N ha^{-1} at sowing. Asterisks denote statistical difference 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.

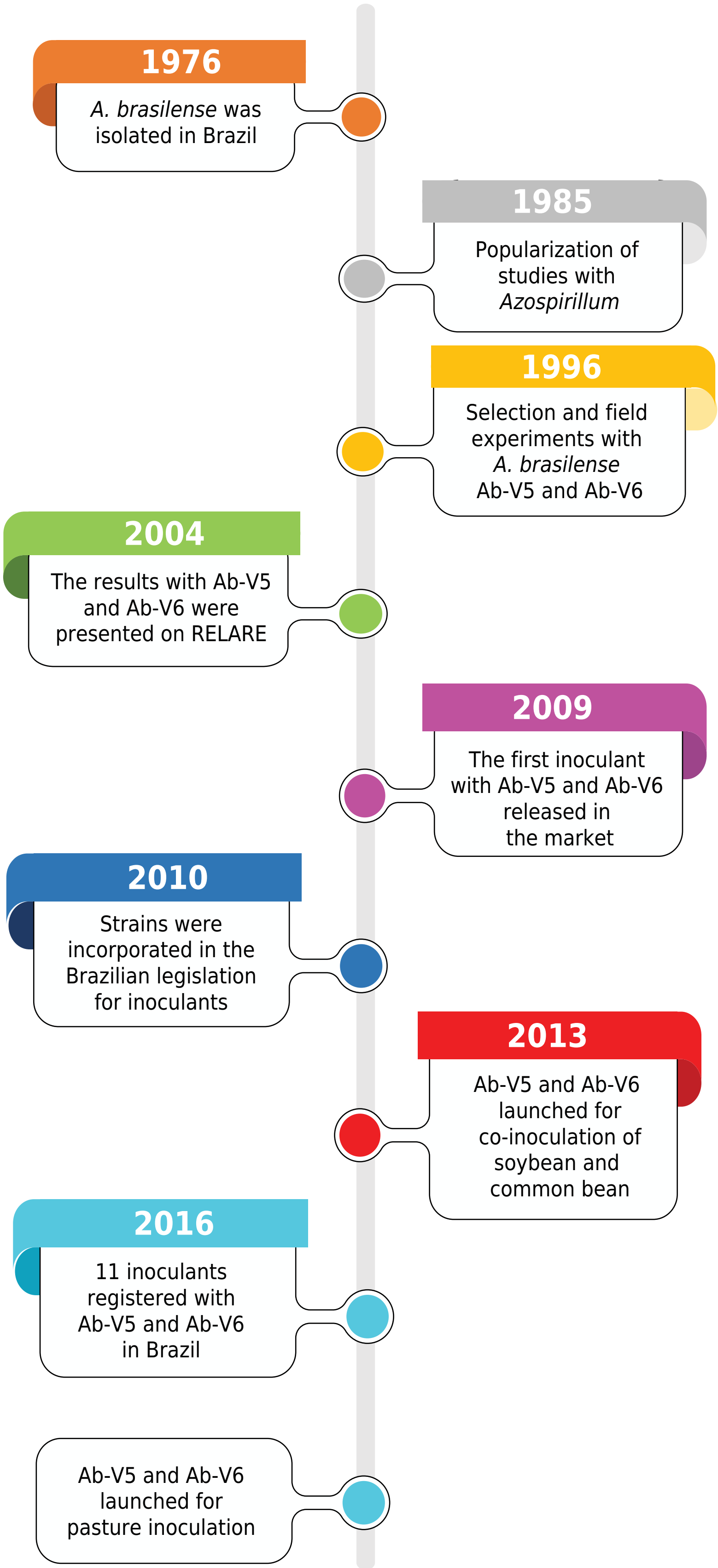

Figure 4. Chronology of some important steps in the prospection, identification, and release of *Azospirillum brasilense* strains Ab-V5 and Ab-V6 in Brazilian agriculture.

ACKNOWLEDGEMENTS

M.S.S. acknowledges a PhD fellowship from Araucaria Foundation of support to the Scientific and Technological Development of the State of Paraná. M.A. Nogueira and M. Hungria are also research fellows of CNPq. We also thank the financial support given by the National Institute of Science and Technology, INCT-Plant-Growth Promoting Microorganisms for Agricultural Sustainability and Environmental Responsibility (CNPq 465133/2014-4, Fundação Araucária-STI 043/2019, CAPES); CNPq-Universal (400468/2016-6), and Embrapa (20.19.02.009.00.01.001).

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