Universidade de Brasília
Instituto de Ciências Biológicas
Programa de Pós-graduação em Biologia Microbiana

# Metabólitos secundários extraídos de rizóbios na suplementação de inoculantes para leguminosas e gramíneas visando melhor desempenho das plantas

Catharine Abreu Bomfim

Brasília – DF
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Tese de doutorado apresentada ao Programa de Pós-Graduação de Biologia Microbiana Instituto de Ciências Biológicas, Universidade de Brasília, como requisito parcial à obtenção do grau de Doutora em Biologia Microbiana.

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**Resumo Geral**

Inoculantes microbianos são produtos que contem na sua formulação microrganismos que atuam de forma benéfica ao crescimento vegetal. Os microrganismos presentes nessas formulações promovem o crescimento vegetal direta ou indiretamente com objetivo de reduzir ou substituir completamente o uso de fertilizantes minerais. Atualmente, esses produtos possuem um grande mercado no Brasil e muitos produtores adotaram o seu uso, especialmente em grandes culturas do agronegócio como soja, milho, feijoeiro e trigo. O grande crescimento populacional aliado a mudanças ambientais tem tornado necessária a maior produção agrícola, porém de forma sustentável. Para alcançar maiores ganhos de produção com o máximo de produtividade agrícola, a pesquisa tem procurado desenvolver formulações microbianas mais eficazes e que garantam maior eficiência da atuação do inoculo. Entre as novas formulações, a suplementação dos inoculantes atualmente aceitos no mercado e contendo estirpes elite com moléculas do metabolismo secundário de rizóbios tem sido alvo de estudos. Inoculantes enriquecidos com extrato metabólico de rizóbios têm mostrado resultados promissores na promoção do crescimento vegetal, mas pouco se sabe sobre os efeitos isolados das moléculas contidas neste extrato, como os lipoquitoligossacarídeos (LCOs), quitoligossacarídeos (COs) e exopolissacarídeos (EPS), quando eles são purificados. Em experimentos conduzidos em casa de vegetação e em campo foi comparado a performance quanto aos aspectos de promoção do crescimento e produtividade de soja, feijoeiro, trigo e milho inoculadas com as bactérias recomendadas para cada cultura e enriquecidas com as moléculas. Os tipos de moléculas e as culturas alternaram quanto a resposta a inoculação acrescida das moléculas. Em particular, a soja e o trigo responderam de forma significativa a suplementação com os metabolitos. Em soja foram observados acréscimos de produtividade em todos os experimentos conduzidos com a cultura inoculada com *Bradyrhizobium* spp. e enriquecida com o extrato metabólico total contendo LCO (EM-LCO). Ganhos de produtividade de 7,6%, 3,1% e 8,6% foram apresentados no tratamento suplementado com EM-LCO em soja em campo e em diferentes safras. Em casa de vegetação, tal qual em campo, o tratamento com EM-LCO aumentou peso seco da parte área e raiz, assim como o número e peso seco dos nódulos em comparação ao tratamento apenas com *Bradyrhizobium* spp. De tal forma, os experimentos com trigo BRS 264 apresentaram resultados igualmente satisfatórios. Essa cultivar, em casa de vegetação, apresentou maior peso seco da parte aérea quando o inoculante a base de *Azospirillum brasilense* foi suplementado com LCO purificado (LCOp) em comparação ao tratamento apenas com *A. brasilense*. Em campo foi observado um aumento de 10% de produtividade do tratamento com *A. brasilense* e LCOp em relação ao tratamento apenas com *A. brasilense*. Entretanto, em milho e no feijoeiro não foi observado diferenças em produtividade e nos demais parâmetros observados dos tratamentos suplementados com as moléculas em comparação com a inoculação padrão recomendada para cada cultura. Possivelmente essa falta de resposta dessas culturas pode estar ligada a cultivar selecionada para o andamento do trabalho e concentração das moléculas. Considerando o exposto, este foi o primeiro trabalho acerca do desempenho agronômico de importantes culturas agronômicas inoculadas com bactérias estirpes e enriquecidas com importantes moléculas de comunicação bactéria-hospedeiro. O desempenho satisfatório da soja e do trigo frente as moléculas sugerem que uma nova formulação de inoculantes microbianos podem ser sugeridas no mercado agrícola brasileiro com a proposta de melhorar a produtividade dessas culturas. Provavelmente há uma correlação do desempenho das culturas com o inoculante e a cultivar, especialmente em gramíneas, além de haver diferenças de dosagens das moléculas entre as culturas, uma vez que foi visto que cada cultura responde de forma individual ao enriquecimento com as moléculas. Esses fatores possivelmente limitaram a resposta do feijoeiro e do milho a suplementação com as moléculas.

**Palavras-chave**: Rizobios, PGPR, fatores de nodulação, *Azospirillum*, *Bradyrhizobium*

**General Abstract**

Microbial inoculants are products that contain in their formulation microorganisms that will act promoting plant growth. The microorganisms present in these formulations promote plant growth, acting directly or indirectly, in order to reduce or completely replace the use of mineral fertilizers. Currently, these products have a large market in Brazil and many producers have adopted their use, especially in crops such as soybeans, common beans, maize and wheat. The large population growth combined with environmental changes has made greater agricultural production necessary, but in a sustainable way. To achieve greater production gains with maximum agricultural productivity, research has sought to develop more effective microbial formulations that ensure greater efficiency in the performance of the inoculum. Among the formulations, the supplementation of inoculants currently accepted in the market and containing elite strains with molecules from the secondary metabolism of rhizobia has been the target of studies. Inoculants enriched with metabolic extract of rhizobia have shown promising results in promoting plant growth, but little is known about the isolated effects of molecules contained in this extract, such as lipochitoligosaccharides (LCOs), chitoligosaccharides (COs) and exopolysaccharides (EPS), when they are purified. In experiments conducted in a greenhouse and in the field, the performance regarding the aspects of promoting the growth and yield of soybean, common bean, wheat and maize inoculated with the recommended bacteria for each crop and enriched with the molecules was compared. The types of molecules and cultures have different responses to standard inoculation enriched with the molecules. In particular, soybean and wheat responded to supplementation with the metabolites. In soybean, were observed a yield increase in all experiments conducted with the treatment inoculated with *Bradyrhizobium* spp. and enriched with the total metabolic extract containing LCO (EM-LCO). Yield gains of 7.6%, 3.1% and 8.6% were achieved in the treatment supplemented with EM-LCO in soybeans in the field and in different crops. In the greenhouse, as in the field, the EM-LCO treatment increased the dry weight of the area and root, as well as the number and dry weight of the nodules compared to the treatment with *Bradyrhizobium* spp. Thus, the experiments with wheat BRS 264 also showed satisfactory results. This cultivar showed higher shoot dry weight when the inoculant based on *Azospirillum brasilense* was supplemented with purified LCO (LCOp) compared to the treatment with *A. brasilense* alone. In the field, an increase of 10% in the yield of the treatment with *A. brasilense* and LCO was observed in comparison to the treatment with *A. brasilense* alone. However, in maize and in common bean, no differences were observed in yield and with others parameters were observed in the treatments supplemented with the molecules in comparison with the standard inoculation recommended for each culture. Possibly this lack of response of these cultures may be linked to the cultivar selected and concentration of molecules. Considering these results, this was the first work of important agronomic crops inoculated with bacterial strains and enriched with important bacteria-host communication molecules. The satisfactory performance of soybean and wheat with the molecules suggests that a new formulation of microbial inoculants can be suggested in the Brazilian agricultural market with the proposal to improve the yield of these crops. There is probably a correlation between the performance of the cultures with the inoculant and the cultivar, especially in grasses, in addition to differences in the dosages of molecules between cultures, since it was seen that each culture responds individually to enrichment with the molecules. These factors possibly limited the response of common beans and maize to supplementation with the molecules

**Key words**: Rhizobia, PGPR, nod factors, *Azospirillum*, *Bradyrhizobium*

**General Index**

**Legends of figures and tables**

**Legend of figures**

**Legend of tables**

**Motivation**

Population growth and food security depend on agricultural practices for food supply and, lately, agriculture has focused on the use of microorganisms to ensure greater agricultural sustainability. Furthermore, microorganisms provide several ecological services necessary for nutrient cycling and act in a beneficial and synergistic way with plants. Modern agriculture has explored the ecological relationships between plants and microorganisms to ensure nutritional supply in replacement and/or reduction of mineral fertilizers and pesticides.

However, agricultural production is still highly dependent on the supply of mineral fertilizers, especially nitrogen. In excess in the soil, nitrogen fertilizers have high mobility, by leaching the nitrate incorporate into water bodies and contaminating watercourses or by transforming urea into $N_2O$, which is a potent greenhouse gas.

The main pillar of the Brazilian economy is agribusiness, which is still very dependent on nitrogen fertilization. Thus, opting for more sustainable food production practices is essential to mitigate the effects caused by the use of agrochemicals. Currently, the ABC plan of the Brazilian federal government has as one of its priorities the strengthening of research, innovation and propagation of technologies that involve the use of microorganisms in agriculture, more specifically, regarding the biological fixation of nitrogen (BNF).

New formulations of inoculants fit within the proposed by the ABC plan to mitigate the emission of greenhouse gases and improve environmental sustainability. Formulations with elite strains containing molecules from the secondary metabolism of rhizobia can potentiate the effects of inoculants already available.

As previously described, the use of beneficial microorganisms in agriculture is a viable alternative to improve agricultural sustainability and new formulations and technologies have been a priority in Brazilian agronomic research. To our knowledge, the development of formulations for large cultures containing molecules from the secondary metabolism of rhizobia is still very incipient, however it is extraordinarily promising.

**Aims and hypotheses**

| | Chapter Title | | Aims | Hypotheses |
|---|---|---|---|---|
| | | **General** | Evaluate the growth-promoting effects of inoculants available on the market for crops of agricultural interest added with secondary metabolism molecules extracted from plant growth-promoting bacteria. | The addition of secondary metabolites extracted from rhizobia will contribute to potentialize gains in yield and growth of inoculants available in Brazil.<br>The conventional inoculation supplemented with LCO and the fractions extracted from the rhizobia will promote gains in growth and yield.<br>The different molecules used will promote plant growth differently for each crop. |
| **Specific** | **Chapter 1** | Brief history of biofertilizers in Brazil: From conventional approaches to new biotechnological solution. | Describe the state of the art about the progress of inoculants in Brazil, considering the research, development and production of these products in the country and shed light on biotechnological innovations. | |
| | **Chapter 2** | Secondary metabolites of *Rhizobium tropici* CIAT 899 added to *Bradyrhizobium* spp. inoculant promotes soybean growth and increases yield | Evaluate the nodular primordia formation in soybean treated with different concentrations of chitooligosaccharide from *Rhizobium tropici* CIAT 899.<br>Investigate the parameters of soybean plant growth promotion in experiments conducted in the greenhouse and in the field with inoculant containing *Bradyrhizobium* spp supplemented with different molecules from *R. tropici* CIAT 899 | Chitooligosaccharide has a biological function in soybean and induces the nodular primordia formation<br>The total metabolic extract containing LCO will increase the performance of soybeans in the field and in the greenhouse.<br>The molecules will promote soybean growth compared to control only with *Bradyrhizobium* spp |
| | **Chapter 3** | Soybean growth promotion of by seed and leaf inoculation with secondary metabolites of *Rhizobium tropici* CIAT 899 | Evaluate the additional foliar inoculation of the metabolic extract containing LCO and exopolysaccharides in soybean enhances the effects observed with the supplementation of the standard inoculant with molecules only via seed. | The additional inoculation of molecules via foliar will differ from the results found with the supplementary inoculation via seeds. |
| | **Chapter 4** | Inoculation enriched with molecules from the secondary metabolism of *Rhizobium tropici* CIAT 899 for growth promotion in common bean (*Phaseolus vulgaris* L.) | Evaluate the formation of nodular primordia in common bean treated with different concentrations of chitooligosaccharide purified from *Rhizobium tropici* CIAT 899. | Chitooligosaccharide has a biological function in common bean and induces the formation of nodular primordia. |

| | | | |
|---|---|---|---|
| | | Investigate the parameters of plant growth promotion of common bean in experiments conducted in a greenhouse and in a field with inoculant containing *R. tropici* CIAT 899 supplemented with different molecules extracted from the same strain | The molecules will promote common bean growth compared to control with only *Rhizobium tropici* CIAT 899. |
| **Chapter 5** | *Azospirillum brasilense* Abv5 and Abv6 supplemented with LCOs extracted from *Rhizobium tropici* CIAT 899 is a promising strategy for formulating a new generation of grass inoculants | Evaluate the response of maize and wheat inoculated with standard inoculant supplemented with rhizobia-legume communication molecules of *R. tropici* CIAT 899 in experiments carried out in a greenhouse and in the field. | Supplementation of the inoculant containing *Azospirillum brasilense* with LCO will promote the growth and increase the yield of maize and wheat |
| **Chapter 6** | Factors affecting the host plant response to inoculation with secondary metabolism metabolites of *Rhizobium tropici* CIAT 899: Final discussion | Conduct a general discussion of all results presented throughout the thesis.<br>Discuss the results and present new perspectives and point out possible paths for study. | |

**Introduction**

The world population in the early 1990s was 5 billion people, reaching 6 billion in 2000 and exceeding 7 billion in 2014. It is estimated that, in the year 2050, the population will be 10 billion people, which makes greater agricultural production necessary to enable global food security (FAO. 2018). Agribusiness in the gross domestic product (GDP) of Brazil is expressive, corresponding with 21% from agriculture. Among the main commodities produced in Brazil is sugarcane (*Saccharum* spp.), soybean (*Glycine max*), maize (*Zea mays*), wheat (*Triticum sativum* L.), rice (*Oryza sativa*), coffee (*Coffea* spp.).

To improve soil fertility and crop yield, modern agriculture uses mineral fertilization. Brazil is highly dependent on fertilizer imports and, therefore, fertilizers and agrochemicals used in Brazil represent the costliest inputs for agricultural production, reaching up to 30% of the final production cost (Olivares et al. 2017). Furthermore, nitrogen fertilizers are associated with pollution of groundwater by nitrates and increased release of greenhouse gases, such as nitric oxide ($N_2O$) - combined with the problem of ozone layer depletion and global warming. Additionally, some mineral fertilizers can cause acidification and imbalance in the soil microbiota (Adesemoye and Kloepper 2009; Calvo et al. 2016; Vejan et al. 2016).

The set of economic and environmental implications that have arisen with the intensive use of fertilizers guide the search for solutions that enable a productive and, at the same time, sustainable agriculture. The use of biological products, which contain microorganisms that promote plant growth, called inoculants, in some countries also called biofertilizers, is one of the main strands in the search for better use efficiency, or complete replacement, of mineral fertilizers.

Microbial inoculants have the potential to provide solutions to agro-environmental problems, because it can improve plant growth and improve soil fertility aspects. (Adesemoye and Kloepper 2009). For over 120 years, microorganisms have been studied using rhizobia in legumes plants. Recently, about half a century ago, another group of beneficial microorganisms became the target of intense studies, plant growth promoting rhizobacteria (PGPR). First described by Kloepper and Schroth (1978), these bacteria comprise a group of beneficial microorganisms that colonize the surface of roots and/or its interior - intercellular spaces - and stimulate plant growth in different ways, being an inseparable part of the rhizospheric microbiota (Hungria et al. 2010; Miransari 2011; Marks et al. 2013; Fukami et al. 2018).

Currently, several products are commercially available, containing microorganisms that are beneficial to plant growth. The main ones in Brazil are those that contain rhizobia for legumes, such as formulations with elite strains of *Bradyrhizobium* spp. for soybeans and *Rhizobium* sp. for the common bean. Furthermore, formulations containing *Azospirillum brasilense*, recommended for the cultivation of maize, wheat, rice and brachiaria, have gained prominence in the Brazilian agricultural market..

Even though inoculation with beneficial bacteria has gained more space in the agricultural market, the use of biological products still accounts for only 5% of the fertilizer and pesticide market in the world. (Arora e Mishra 2016), far below what is needed for greater productive sustainability. Thus, new technologies are needed to make these products more effective in the field, less costly and gain more market among producers. Currently, several studies are being carried out regarding formulations such as the use of secondary metabolites in combination with beneficial bacteria.

Among the metabolites that have gained prominence, the nodulation factors or lipochitooligosaccharides (LCOs) have been more explored. LCOs act as signal molecules that have high biological activity, inducing host responses at submicromolar concentrations between $10^{-9}$ and $10^{-12}$ M. In

most legumes, LCOs, is essential molecules for host-symbiont specificity, also can act in root deformation, alter the flow of ions within the host cell, participate in intra- and extracellular alkalinization, in the formation of the infection cord and in the nodule organogenesis, through stimulation of cortical cell division in the plant and activation of genes involved in nodulation (Macchiavelli and Brelles-Mariño 2004; Oldroyd 2013; Pérez-Montaño et al. 2014a; Gourion et al. 2015; Schwinghamer et al. 2015; Kaschuk and Hungria 2017; del Cerro et al. 2019).

Nodulation factors act on several physiological processes in the host in addition to those involved with nodulation, such as the formation of lateral roots (Oláh et al. 2005). Furthermore, LCOs activate the expression of genes involved in the plant cell cycle, stimulating cell division, not only in legumes and, due to this, stimulate germination, seedling growth and root growth in several non hosts, when applied to seeds (Prithiviraj et al. 2002; Kidaj et al. 2012) and the increase in leaf area, increase in photosynthetic rate and total dry weight when applied via the foliar (Khan et al. 2008). Souleimanov et al. (2003) observed that at submicromolar concentrations ($10^{-7}$ to $10^{-9}$ M) the purified LCO from *B. japonicum* 532C improved the germination of maize, rice, soybeans, beans and greater accumulation of biomass, supporting the theory of hormone-like action of these molecules proposed by Fisher e Long (1992).

The additional addition of communication molecules, such as flavonoids and nodulation factors, together with the recommended inoculants for legume crops, act by enhancing the action of these products. Maj et al. (2010) observed that the nodA gene – encodes the primary structure of the LCO – of *R. leguminosarum* was induced in the presence of flavonoids and root exudates. Pre-induction of *R. leguminosarum* bv. *trifolii* with *Trifolium pratense* exudates increased the number of nodules and dry mass of the aerial part when used as an inoculant in *T. pratense*.

Nodulation factors combined with the use of host-specific rhizobia are beneficial to plant growth in both legumes and non-legumes. Purified LCOs, in the absence of rhizobia, are sufficient to induce root hair bending, cell division and the formation of nodule-like structures (Skorupska et al. 2010). López-Lara et al. (1995) added purified LCO from *Rhizobium* sp. GRH2 in *Phaseolus* sp. and *Acacia* spp. and observed that purified LCO induced root hair formation and deformation. In a similar study conducted by Stokkermans and Peters (1994) there was the formation of nodule-like structures in the root hairs in *Glycine max* treated with LCO extracted from *B. elkanii*.

Furthermore, other molecules involved in host-microorganism communication and also in biofilm formation can act beneficially when supplemented in inoculant formulations, such as exopolysaccharides (EPS). EPS is the main structural component of biofilms, being responsible for the architecture, stability and organization of the cell cluster in micro-colonies. The structural components of the biofilm provide resistance to desiccation, passive absorption of nutrients from the adhesion region, act as a carbon source and resistance to biotic stress conditions (protection against antimicrobial compounds and toxins) and abiotic stress (changes in pH and variations in temperature) (Flemming et al. 2016). EPS has elucidated effects on the structure of bacterial micro-colonies and it is currently known that host plants recognize these molecular patterns and exhibit physiological responses (Fuqua et al. 1994; Whitehead et al. 2001; Ortíz-Castro et al. 2008; Pérez-Montaño et al. 2011).

New bioformulations, such as inoculants containing molecules, use molecules from the secondary metabolism of beneficial bacteria in order to increase the response of the plant. Molecules involved in plant-

bacteria communication, such as LCOs, and in bacteria-bacteria communication are currently being studied to compose these new formulations of molecular inoculants.

The main objective of this work was to evaluate the response of the main crops of agricultural interest with inoculants available on the market. The inoculants for soybean, common bean, maize and wheat were enriched with molecules important for rhizobia-legume communication and important for bacterial biofilm formation, EPS.

# Chapter 1 - Brief history of biofertilizers in Brazil: From conventional approaches to new biotechnological solutions[1]

"*A pesquisa deve ser feita com sensibilidade e criatividade, sempre buscando novos caminhos para o bem estar de nossos semelhantes*"

*Dra. Johanna Dobereiner*

**Abstract**

Brazil has a long history of research with rhizobia and plant growth promoting rhizobacteria (PGPR). Currently, the use of bio-based products in Brazil, containing microorganisms that are effective in promoting plant growth through various mechanisms, is already a consolidated reality for the cultivation of several crops of agricultural interest. This is due to the excellent results obtained over many years of research, which contributed to reinforce the use of rhizobia and PGPR by farmers. The high quality of the products offered, containing elite strains, allows the reduction and prevention in the use of mineral fertilization, contributing to low-cost and sustainable agriculture. Currently, research has turned its efforts in the search for new products that further increase the efficiency of those already available on the market and for new formulations or inoculation strategies that contribute to greater productivity and efficiency of these products. In this review, the history of biological products for main crops of agricultural interest and the new biotechnologies and research available in the agricultural market are discussed.

**Keywords**: Rhizobia, PGPR, Inoculant, *Azospirillum*, *Bradyrhizobium*

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## 1 Introduction

It is estimated that, in the year 2050, the world population will reach the mark of 10 billion people, which makes greater agricultural production necessary to make the population's food security feasible [1]. Currently, Brazil occupies the fifth place among the countries that have the largest territorial extension focused on agriculture, with about 7.8% of its territory used for agricultural practices [2]. In Brazil, the agribusiness participation in the Brazilian GDP (Gross Domestic Product) was 26.6% in 2020, within this amount the agricultural sector contributes with 70%, with grain production being the main one [3]. In order to guarantee high productivity in the agricultural sector, chemical fertilizers are widely used by agricultural producers, which increases the final cost of production by up to 30% [4]. In addition, improper handling of chemical fertilizers can negatively affect the environment and the soil. Nitrogen fertilizers, for example, are associated with soil biota acidification and imbalance, pollution of groundwater by nitrates and increased release of gases that participate in the greenhouse effect, such as nitrous oxide ($N_2O$), combined with ozone layer depletion and global warming [5–7].

The set of economic and environmental implications that have arisen with the intensive use of fertilizers guide the search for solutions that enable a productive and, at the same time, sustainable agriculture [8,9]. The use of biological products, which contain microorganisms that promote plant growth, called inoculants, in some countries also called biofertilizers, is one of the main strands in the search for better use efficiency, or complete replacement, of mineral fertilizers.

In Brazil, microbial inoculants are defined as a product that contains microorganisms that are beneficial to plant growth [10] which are equivalent to the biofertilizers internationally [5,11]. The main inoculants produced and commercialized in Brazil are currently formulated with bacteria called rhizobia, which comprise a group of species that establish a symbiotic relationship with plants of the Fabaceae family. This interaction has been known for over 120 years and it forms structures called nodules, where the biological nitrogen fixation (BNF) occurs [11]. The use of these bacteria in agriculture can supply, totally or partially, the nitrogen needs of several leguminous plants.

There are other nitrogen fixing (diazotrophic) bacteria that are free living or associated with plants, without the formation of nodules, also provide nitrogen to the plant, but less efficiently. Bacteria of the genera *Azospirillum*, *Pseudomonas*, *Bacillus*, *Azotobacter* among others, are called plant growth-promoting rhizobacteria (PGPR) and are used in inoculant formulations to directly assist plant growth by improving the acquisition of nutrients by the plant through various mechanisms, such as mineralization and solubilization, BNF and synthesis of growth regulators (e.g., indole acetic acid, cytokinin), which act directly on root, increasing the root-soil contact surface, allowing greater absorption of water and nutrients and volatile organic compounds (VOC) which has an antifungal activity [12]. Indirectly, the PGPRs or the products of their metabolism, can act as biological control agents and induce systemic resistance. Also, the production of the ACC deaminase enzyme acts controling plant stress, decreasing the ethylene levels by cleaving its precursor and reducing its production, providing resistance to crops under abiotic stress conditions, acting on hormonal metabolic pathways in the plant [12–14] (Figure 1).

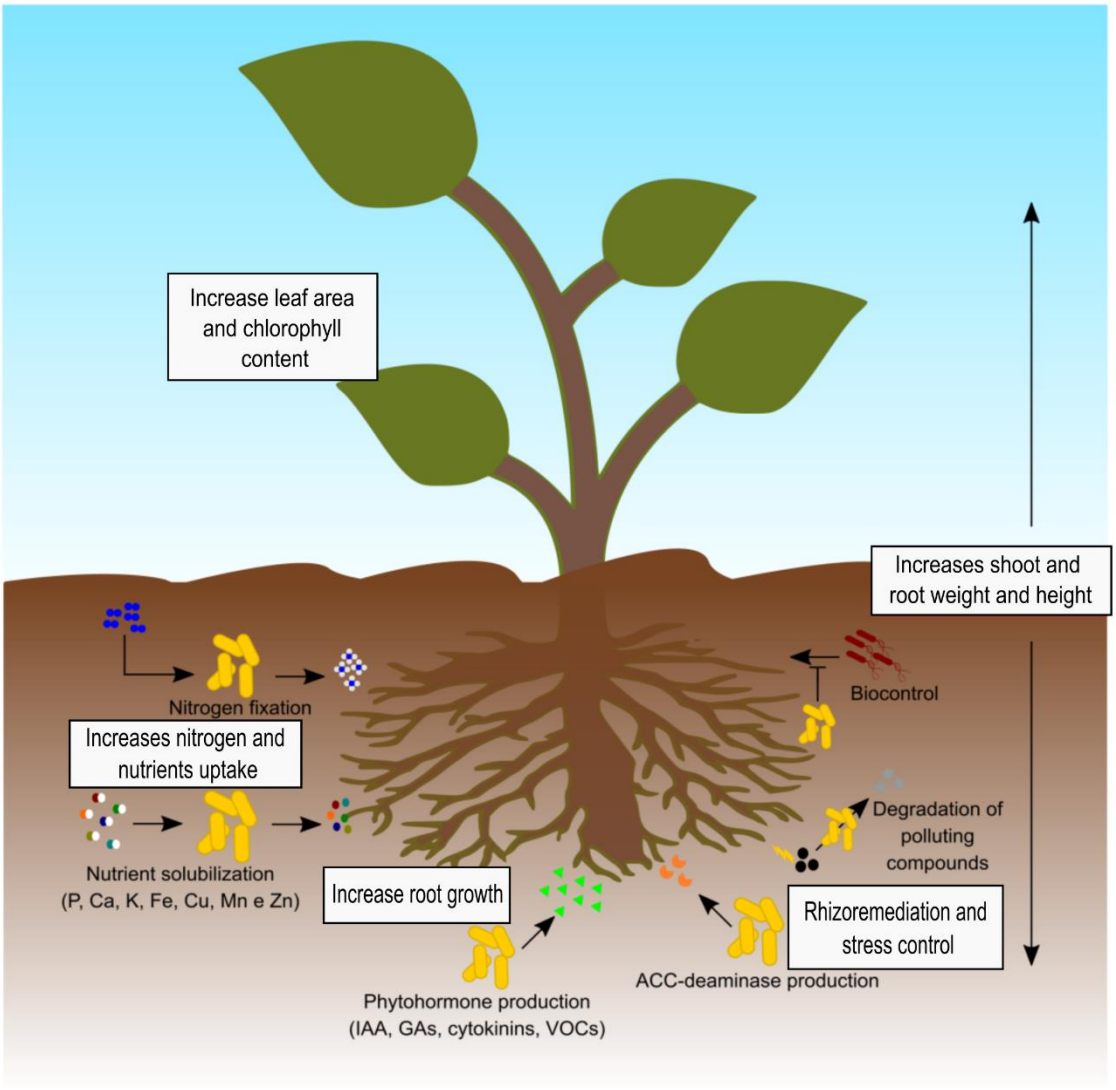


**Figure 1**. Direct and indirect mechanisms for promoting plant growth by PGPR. In red, the benefits to plants of plant-PGPR interaction

In general, studies with growth-promoting microorganisms focuses on isolating and characterizing new PGPR [15–20] and evaluate the performance of these microorganisms in plant development in order to produce new formulations [21–23]. In addition, technologies that improve the use of microorganisms in agricultural processes have been studied [24] such as the combined use of two or more microorganisms [25–30], use of secondary metabolism molecules [31,32] and new inoculants formulations, which aim at the greater effectiveness of these products. The objective of this review is to present a brief history of the use of inoculants in Brazil, focusing on the main products for crops of agricultural interest, and present new researches and technologies that have been developed to increase the quality and efficiency of inoculants already available.

## 2 Bacteria of agricultural interest: History and use in Brazil.

The first studies in Brazil. with rhizobia of agricultural interest took place in the state of Rio Grande do Sul to select strains for clover (*Trifolium* spp.) and alfalfa (*Medicago sativa*) crops intended for livestock production [33]. In the early 1960s, when soybean (*Glycine max* L. Merr]) production started its expansion in Brazil [33] the industry of rhizobia based products also followed the same trend.

A decisive milestone for the production of commercial inoculants was the research which prioritized the search for strains chosen in the inoculation of soybeans and other crops of agricultural interest [34]. Brazil has a long history of research with inoculants and it is impossible not to mention the fundamental role that great researchers such as Johanna Döbereiner and João Ruy Jardim Freire played in this scenario [34,35]. According to Döbereiner [34], the soybean crop has become a highlight in the Brazilian agricultural panorama, with the use of nitrogen fertilization being entirely dispensed, due to the joint work of microbiologists and breeders. Another decisive factor to guarantee the production and wide acceptance of commercial inoculants in Brazil was the creation of a legislation that establishes criteria with regard to specifications, guarantees, registration, packaging and labeling of the inputs intended for agricultural use, in addition, it lists the authorized and recommended microorganisms for the production of the inoculants [36]. The first law which defined the commercial standards for this type of product appeared in 1980, and since then has been updated in several occasions. According to the most recent update, about 118 strains of rhizobia are authorized for the production of inoculants for legumes and 12 PGPRs recommended for other crops such as rice, wheat, corn and eucalyptus [10].

The companies that produce inoculants in Brazil must accomplish a series of requirements with regard to concentration, purity, shelf life guarantee and absence of biological contaminants in the products. Nowadays, the production of crops of agricultural interest such as soybeans, common beans, maize, wheat, sugar cane, including brachiaria pastures, have biological products that are recommended for inoculation with wide acceptance.

### *2.1 The case of soybean in Brazil.*

Soybean (*Glycine max* (L.) Merr.) is a leguminous plant of the Fabaceae family, originally from East Asia used as a staple food in the eastern civilization. Only in the 1960s soybean become economically important in the Brazilian scenario, developing a larger area of cultivation and investment in technologies that resulted in yield increase [37]. The expansion in the agricultural area for soybean cultivation started from 6.8 million hectares in the 1970s to 38.26 million hectares in 2021 [2]. Currently, soybean production is among the most profitable agricultural activities in Brazil, which is the largest producer and exporter in the world. The BNF has always been a priority in the soybean planting system in the country. Since the entrance of this crop in Brazil, research has been focused on the identification and selection of strains of *Bradyrhizobium* with symbiotic superiority. However, Brazilian soils, at the beginning of soybean cultivation in the country, were free of *Bradyrhizobium* and due to the absence of the natural diversity of these microorganisms the first inoculants used were imported from other countries. Subsequently, the search for variant genotypes with high capacity for $N_2$ fixation and more adapted to the Brazilian soils and environmental conditions began using techniques for re-isolating rhizobia from soybean nodules [38].

The research with rhizobia capable of nodulating soybean began in the 1940s with the selection of strains, tests with imported inoculants and attempts at re-isolation [36]. However, it was only in the 50s that the selection of effective strains in soybean nodulation and the production of inoculants was actually carried out [38]. The pioneering work of the researchers Johanna Döbereiner and Jardim Freire in the search for more competitive and effective strains for this crop was essential for the independence of nitrogen fertilizers in soybean cultivation. The work of these researchers was also decisive for the soybean breeding programs to be carried out in the absence of nitrogen fertilizers, considering only the symbiosis of this plant with the rhizobia [36].Due to the intense search for superior rhizobia genotypes, Brazil is currently one of the largest producers of inoculants in the world and the consumption of this technology has a high acceptance by great part of the farmers [38–40]. The expansion of soybean cultivation to the Cerrado in the 1980s, started with the selection of elite rhizobia strains capable of meeting plant's demand for nitrogen in low humidity conditions and in acidic soils. The first strains selected for soybean inoculation in the Brazilian Cerrado were *Bradyrhizobium elkanii* SEMIA 587 and SEMIA 5019 (= 29w) [41]. These strains, possessed a high capacity to fix nitrogen, good competitive ability and were able to nodulate a wide variety of soybean cultivars [41]. The inoculation success with these strains in the 1980s among other advances in crop production system contributed to increase the agricultural frontier in the country and, as a result, soybean became the most important agricultural crop in Brazil [40].

Peres et al. [42] presented the strains of *B. japonicum* SEMIA 5079 (= CPAC 15) and *B. diazoefficiens* SEMIA 5080 (= CPAC 7), as highly competitive and with high nitrogen fixing potential [38,39,43]. The inoculation of soybeans with SEMIA 5079 and SEMIA 5080 showed gains in productivity 8.8% higher than those presented using the strains of *B. elkanii* SEMIA 5019 and SEMIA 587 [40]. In 2019, a consumption of 70 million doses of soybean inoculants was estimated, used in more than 90% of the total planted area in Brazil [44]. Soybean inoculation allows an average yield of 3.5 tons of grains $ha^{-1}$, without the need of nitrogen fertilizers [45]. On the other hand, only 15% of the total area used for planting soybeans in the USA are inoculated [46,47]. This is due to the low cost of nitrogen fertilizers and low incentives in the use and development of biological based technologies in that country [36].

Considering the reduction in the total spending on nitrogen fertilization for the crop and the wide acceptance of the technology by producers, an annual savings of US$15 billion in Brazil is calculated, allied to a significant reduction in the emission of greenhouse gases and groundwater contamination [44]. The calculation of savings in nitrogen is carried out as described by Hungria and Mendes [48], which considers the size of the total planted area in the country, the price of the nitrogen (urea) and the average yield per hectare. Furthermore, this calculation also considers a 50% nitrogen use efficiency and the estimate of the total N exported by the crop. Assuming that the use of nitrogen in soybean cultivation is unnecessary, it is possible to assume that the calculated amount that would be spent on urea is saved with the use of rhizobia in soybean inoculation.

Between 2009 and 2018 the use of inoculants for soybean has increased to 221% [49], used both domestically and for export to countries in South America and Africa, with the vast majority containing the elite strains SEMIA 5079 and SEMIA 5080 [38]. Currently, new ways of inoculation and improvement of the formulation have been studied to improve its efficiency, and in return, increase the demand for this type of product in agriculture.

### *2.2 Common bean*

Common bean (*Phaseolus vulgaris* L.) is a legume from the Fabaceae family grown in different regions of the world. Brazil is one of the largest producers and consumers of this legume, which is widely consumed in South America and Africa. Currently, about 2.9 million hectares in Brazil are used for the common beans cultivation, in three annual harvests, reaching a production of 3 million tons. However, the average yield of each crop is 1000 kg $ha^{-1}$, well below its productive capacity [2]. Environmental stresses such as drought and low nutrient supply are limiting factors for the development of the plant and, common causes, for the common beans low productivity [27,50].

Common bean is considered a host for the rhizobia with low specificity, or promiscuous, capable to form nodules with different genera and species of bacteria of the alpha-proteobacteria group (mainly *Rhizobium*) and beta-proteobacteria (as *Paraburkholderia nodosa*, *P. tuberum*, *P. sabiae* and *Cupriavidus necator*). The most common bacterial species capable of nodulating common bean belong to the genus *Rhizobium*, such as *R. tropici* [51], *R. etli* [52] *R. freirei* [53], *R. leucaenae* [54], *R. paranaense* [55], among others. In Brazil there are three bacteria of the genus *Rhizobium* authorized for commercialization in inoculant formulations and recommended for common beans cultivation, the *R. tropici* SEMIA 4077 (= CIAT 899) and SEMIA 4088 (= H12) and *R. freirei* SEMIA 4080 (= PRF 81). These bacteria were selected mainly for having greater genetic stability and high tolerance to environmental stress conditions [56,57].

Due to a series of factors that affect the efficiency of BNF in common beans and the great demand for nitrogen by the crop, the results in productivity can be variable, being necessary, in some cases, a suplementation with nitrogen fertilizers to reach high production levels [58,59]. The main reported factors involve early senescence of the nodules [60], genotype-specific interaction between bacteria and host plant [61,62] and abiotic factors, mainly water deficit [63], soil fertility and temperature [64]. The presence of highly competitive native rhizobia capable of nodulating the common bean (but with a low fixative capacity) is one of the main causes of the low efficiency of BNF in this crop [65,66] and can affect inoculation responses with elite strains [67].

In the state of Paraná, inoculation of common beans with *R. tropici* CIAT 899 and *R. freirei* PRF81 resulted in productivity above 2.500 kg $ha^{-1}$ without the addition of N fertilizer [56,57]. In the Midwest region, common bean production may still depend on complementary doses of N fertilizers, along with inoculation to achieve high levels of productivity. According to Pelegrin et al. [68], common bean inoculation was equivalent to a fertilization of 80 kg of N $ha^{-1}$ in the state of Mato Grosso do Sul. In addition, when 20 kg of N $ha^{-1}$ was added in the seeding, associated with the inoculation with *R. tropici* CIAT 899, a yield of 3.339 kg of grains $ha^{-1}$ was obtained, equivalent to a fertilization with 160 kg of N $ha^{-1}$ [68]. Depending on the cultivar, the productivity of beans can vary from 840 kg $ha^{-1}$ to 2.741 kg $ha^{-1}$ of grains, when inoculated with elite strains recommended for the crop [61,62].

Based on the promising results of the use of commercial inoculants in common bean crops and, mainly, in the possibility of reducing production costs, the sale of this biological input increased by 85% between 2009 and 2013 [49], and only in 2018, approximately 280 thousand doses of inoculant for beans were sold in peat and liquid formulations [49]. Strategies, such as co-inoculation with *Azospirillum brasilense* [69] and *Bradyrhizobium* spp. [26], nitrogen fertilization associated with elite strain inoculation [68] and the search for new elite bacteria [70], are the subject of studies for common bean.

***2.3 Grasses.***

The cultivation of cereals started thousands years ago and, until the present moment, represents the basis of the world nutrition. Among the main crops, wheat, corn and rice stand out as important cereals for human and animal food [71]. Cultivated pastures are also an economical and viable agricultural practice for cattle feed, in addition it is an important practice for the recovery of degraded areas [72]. The cultivation of grasses is dependent on mineral fertilization, especially with nitrogen, to achieve high yields. In Brazil, there is a long history of research with PGPR associated with grasses, especially for pioneering work conducted by Dr. Johanna Döbereiner, which aimed at reducing mineral fertilization and guaranteeing the maximum production of crops with agricultural sustainability [73–75]. Among the most studied microorganisms, bacteria of the *Azospirillum* genus stand out as PGPRs for a wide variety of host plants, many of great economic importance, such as corn and wheat [75].

Bacteria from the *Azospirillum* genus are alpha-proteobacteria, which can be free-living or associated with the rhizospheric region and/or endophytically in the colonization of more than one hundred hosts [76]. This bacterial genus was first described by Tarand [77], with *A. brasilense* and *A. lipoferum* species. Currently, a total of 22 species have been described, isolated mainly from the soil, with worldwide distribution[78].

The first studies carried out with this bacteria aimed to assess its ability to fix atmospheric nitrogen and reduce the use of mineral N, especially in grasses [73,79]. Its first species was described by Beijerinck in 1925 and it was called *Spirillum lipoferum*, but it was only in 1978 that it was discovered that this bacterium has the ability to fix atmospheric nitrogen, having its scientific name changed to *Azospirillum lipoferum* [77]. Shortly after, Tien et al. [80] reported the production of several growth regulators by *Azospirillum*, such as auxins, gibberellins and cytokinins. It is now known that the benefits of inoculation with this bacterium go beyond BNF, which reinforces the theory of Bashan and Levanoy [81] about the "additive hypothesis", that considers the involvement of multiple mechanisms of action in the association of *Azospirillum* with the host. The hypothesis suggests that the mechanisms operate simultaneously or in association, with the contribution of one of the mechanisms being less effective when evaluated separately.

The inoculation with the *A. brasilense* species benefits the plant in several ways, such as the induction of systemic resistance, inducing the synthesis of a variety of secondary metabolites by the host [82]. Furthermore, the association among plants and *A. brasilense* can provide protection from abiotic stress conditions, such as salt and oxidative stress [83,84] and the production of growth regulators by bacteria results in morphological changes in the roots, promoting greater root growth and resulting in better absorption of nutrients and water [80,85–89]. In the case of seed inoculation, the growth regulators produced by *Azospirillum* in the product act in "seed primming" effect, that is, after inoculation, the number of viable bacterial cells is drastically reduced, however, the concentration of growth regulators remain promoting plant growth [78].

Several studies point out the beneficial effects of inoculation with *A. brasilense* in grasses of agronomic interest such as corn (*Zea mays* L.), wheat (*Triticum aestivum* L.) [75,90,91], rice (*Oryza sativa*) [88] and brachiaria (*Urochloa* spp.) [72]. Hungria et al. [75] evaluated the inoculation of *A. brasilense* Abv5 and Abv6 in wheat and corn by conducting 17 experiments carried out in different stations located in nine areas with different edaphoclimatic conditions. There was an increase in nutrients concentration in the grains and a greater root mass in the inoculated plants, with an increase of 24-30% and 13-18% in corn and

wheat productivity, respectively. Díaz-Zorita and Fernández-Canigia [92] found similar results in 297 experiments conducted in Argentina with wheat, using *A. brasilense* Az-39 and positive responses were observed in 70% of the experimental areas, with an 8% gain in grain yield. Okon and Labandera-Gonzalez [86] in experiments carried out in several countries with *A. brasilense* also reported a 5-30% increase in grain yield in 70% of the evaluated areas.

In Brazil, the inoculants commercialization containing the strains of *A. brasilense* Abv5 and Abv6 has been carried out since 2009, for wheat, rice, corn [75] and, more recently, for brachiaria [72]. In 2018, more than nine million doses of inoculants with *A. brasilense* were commercialized, accounting for approximately 10% of the total inoculants sold in Brazil, indicating the great acceptance of this bioproduct by producers [49].

The *Nitrospirillum amazonense* species, isolated from sugar cane and other grasses such as corn, sorghum and rice is widely distributed in the Brazilian territory [93]. Possibly, its wide occurrence in the Brazilian soils is associated with high adaptability to acidic soils, a very common characteristic in most of the country's soils. [94,95]. Recently a public-private partnership between the Brazilian Agricultural Research Corporation (EMBRAPA) and BASF company announced a new inoculant that has this bacterial species in its formulation and it is recommended for the cultivation of sugar cane. BASF is commercializing this inoculant associated with other products of insecticidal and fungicidal action, with the name of 'Muneo Bio' Kit.

Even though inoculation with beneficial bacteria such as rhizobia, *Azospirillum* spp., *Bacillus* spp. and *Pseudomonas* spp. have gained more space in the agricultural market, the use of bioproducts still accounts for a small share of the fertilizers and pesticides in the world, far below what is necessary for greater productive sustainability [96]. New technologies are needed to make these products more effective in the field, less costly and more attractive to producers. Currently, several studies are being carried out with regard to new formulations of beneficial bacteria and the use of secondary metabolites in combinations with them.

### *2.4 Phosphate solubilization.*

Phosphorus is one of the most limiting elements for plant growth and decisive for agricultural crops productivity. Tropical soils used in agriculture are generally acidic, with the presence of phosphates, especially iron phosphate ($FePO_4$) and aluminum phosphate ($AlPO_4$), that are unavailable to plant metabolism [97]. The lack of this nutrient is supplied with the application of phosphate fertilizers. High doses should be applied to the soil, due to its low efficiency, which makes the use of phosphate fertilization costly to the producer, especially in Brazil, which has a high dependence on the import of this input [98].

Phosphate solubilizing microorganisms (PSM) convert the sparingly soluble phosphate to its soluble form, or assimilable, to plant metabolism through the production of compounds capable of breaking the phosphate bond with its chelating agent. Among the main forms of action of these microorganisms are the production of organic acids, siderophores, protons and CO2 [98–100]. Several microorganisms, including bacteria and fungi, have the ability to solubilize phosphates, such as *Aspergillus*, *Penicillium*, *Bacillus*, *Pseudomonas* and *Paraburkholderia*. Many studies have focused on the prospecting of PSM for the production of inoculants [99].

Several microorganisms are marketed as PSM in the world, one of the main is the *Penicillium bilaiae* fungus, commercialized in Canada in a formulation called JumpStart XL® (Bayer), recommended for use in various cultures. In field, the use of this product increased productivity, P content and reduced phosphate application in wheat, in addition to contributing to greater phosphate absorption in other crops [101]. Currently, many researches have focused on the combined use of PSM with other microorganisms such as rhizobia [102] and mycorrhizal.

Oliveira et al. [103] described 45 PSM isolated from the corn rhizosphere grown in an area of the Brazilian Cerrado with low phosphate concentration in the soil, the authors report that the isolates *Bacillus* sp. (B17), *Burkholderia* sp. (B5) and *Streptomyces platensis* (A4) were the most efficient in solubilizing calcium phosphate $Ca_3(PO_4)_2$, promising for the formulation of an inoculant. Ribeiro et al. [97] found that strains of *Bacillus* B1923, B2084 and B2088 promoted foliar and root growth and accumulation of macronutrients in millet, independently, varying according to the inoculated strain and source of phosphate present. These microorganisms have other characteristics in promoting growth, such as the production of growth regulators and siderophores. The isolates B2084 and B2088 produced gluconic acid in vitro and it is recognized by literature as one of the most efficient mechanisms in the solubilization of phosphate by bacteria.

These studies contributed to the development of the first commercial inoculant based on phosphate solubilizing bacteria in Brazil, composed of *Bacillus megaterium* CNPMS B119 and *B. subtilis* CNPMS B2084, BiomaPhos® (Bioma), the result of research by the Brazilian Agricultural Research Corporation (Embrapa) with the company Bioma [104]. Paiva et al. [104] show that the combined use of these two strains was effective in increasing corn productivity. The authors carried out several experiments, in three harvests, in different locations in Brazil and the average results of productivity gain with inoculation were 8.9%, resulting in average yield of 10 bags of corn grains per hectare. The use of this product in the cultivation of soybeans was also effective in increasing productivity, resulting in an average increase of 5 bags per hectare [101]. Currently, research with these same strains continues to be carried out with other cultures.

The production of phosphate fertilizers is carried out from non-renewable sources, generating environmental impacts. Furthermore, Brazil is still dependent on imports of this product, although there is an enormous reserve of phosphorus that cannot be assimilated in the soil. The development of inoculants based on PSM is extremely important to reuse the phosphate stock fixed in the soil, chelated in clay or metals (Al, Fe and Ca, especially) and reduce the use of phosphate fertilizers.

### *2.5 Co-inoculation.*

The combined use of different PGPRs that work through different mechanisms of action promoting plant growth is a strategy that has been widely explored in the inoculation of different cultures [69,105]. The combination of two or more microorganisms has already been used in agriculture, such as the combination of rhizobia with phosphate solubilizing bacteria [106], PGPRs with another PGPR that acts as a pathogen biocontrol agent; or bacteria that have the same mechanisms of action in promoting growth, but with differences in tolerance to abiotic conditions of the medium or specificity to the plant's genotype [8,107].

In Brazil, there has been success in the combined use of rhizobia strains recommended for soybeans and common beans with *A. brasilense* Abv5 and Abv6 [69,108]. The inoculation of rhizobia with *A. brasilense* increased the productivity in 16.1% and 19.6%, in soybean and common beans, respectively, when compared with the controls inoculated only with rhizobia [69]. The combined use of rhizobia and *A. brasilense* benefits the plant in increasing the number of nodules [109,110], greater tolerance to environmental stress conditions [83], and it is an economically profitable practice for the producer. Galindo et al. [25] demonstrated that the inoculant based on *A. brasilense* represents only 1.1% of the total operational cost of soybean cultivation, however, productivity gains are approximately 10 bags of grains per hectare compared to cultivation with only *Bradyrhizobium* spp.

The co-inoculation of rhizobia and *A. brasilense* AbV5 and AbV6 is a practice recommended for soybean and common beans producers in Brazil and, currently, co-inoculation is already a reality for most farmers. Between 2015 and 2018 there was an increase of 220% in the commercialization of inoculants composed by *A. brasilense* [49]. Although the increase in the commercialization of this product is due to grasses cultivation, a large part of the sales is destined to the combined use of inoculants recommended for legumes [36]. Currently, some inoculant companies in Brazil already offer for sale a single product containing *Bradyrhizobium* spp and *A. brasilense* for soybean inoculation.

The combined use of five strains of PGPR is recommended for sugarcane in Brazil. This formulation is composed by *Gluconacetobacter diazotrophicus* (BR 11281), *Herbaspirillum seropedicae* (BR 11335), *Herbaspirillum rubrisubalbicans* (BR 11504), *Nitrospirillum amazonense* (BR 11145) and *Paraburkholderia tropica* (BR 11366). All bacteria present in the formulation are diazotrophic and phytohormone-producing, and isolated from sugar cane [111]. Co-inoculation with the five strains is recommended in order to reduce the difference in response among distinct sugarcane cultivars [112].

Co-inoculation offers several benefits to the plant, such as an increase in root area, which enables a greater use of mineral fertilization and water absorption, ensuring protection from water stress conditions, and provide legumes with greater root surface for rhizobia infection, increasing nodules formation [25]. It is the target of several studies that pursuit to deepen the knowledge about bacteria that can be the object of new formulations, compatibility of different strains and use in different cultures.

## 3.0 New inoculant formulations.

### *3.1 Molecular inoculants: addition of secondary metabolism molecules in formulations already consolidated on the market.*

#### *3.1.1 Nodulation factors (Nod factors).*

The process of forming active nodules in leguminous plants is extremely complex and depends on the communication between the host plant and the rhizobia through signal molecules. The formation of functional nodules is initiated by the production of phenolic compounds by the host, such as flavonoids, which act as an inducing molecule [113,114]. These compounds, in addition to acting as chemotactic agents for rhizobia, activate the transcription of nodulation genes (*nod* genes) and promote the production of signal molecules by rhizobia called lipochitooligosaccharides (LCOs), or nodulation factors (NF), essential for the specificity of host-symbiotic communication [12,115,116]. Nodulation factors play a crucial role in the formation of active nodules and in submicromolar concentrations, among $10^{-9}$ and $10^{-12}$ M [117], they induce physiological and morphological changes in the host such as alteration of the ions flow, resulting in

the root hair curling and formation of the infection cord that allows the rhizobia to enter the cells. In addition, nodulation factors promote the nodular primordium formation, which after the rhizobia infection and differentiation, forms the active nodule (Figure 2) [8,114,118–121].

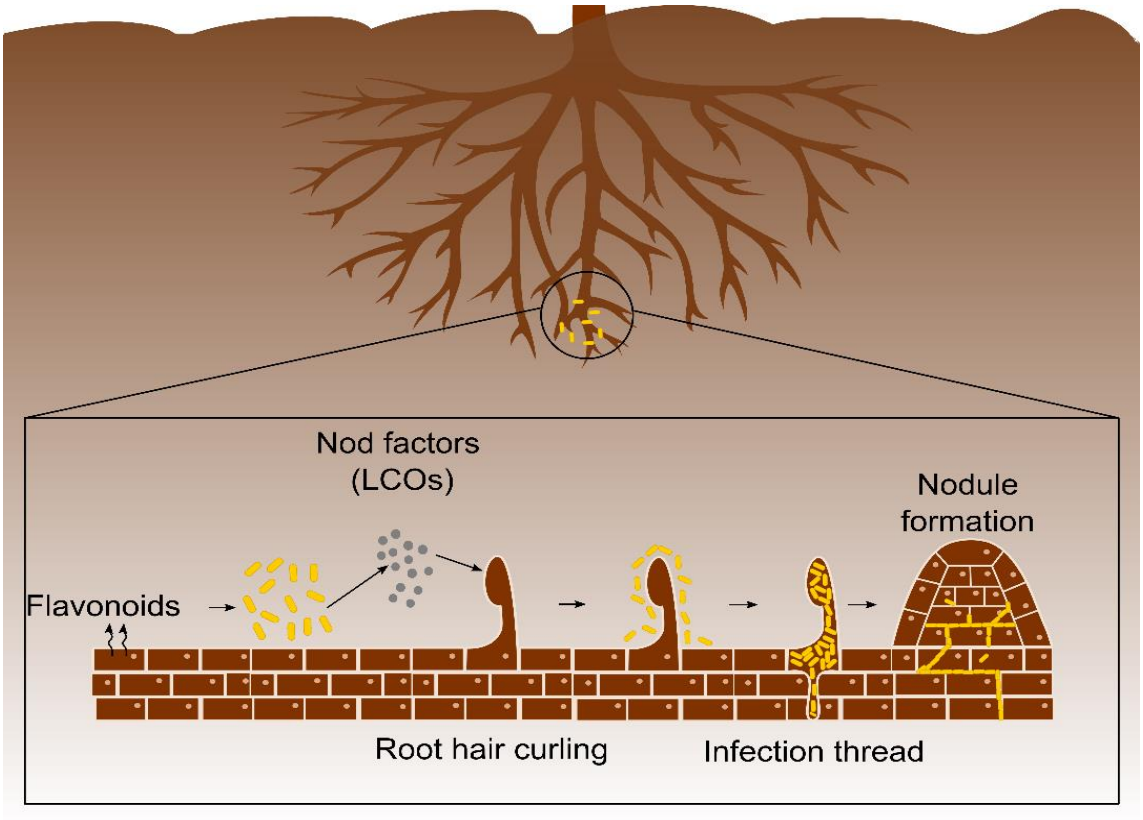


**Figure 2.** Colonization of rhizobia and nodule formation**.** Flavonoids are produced by the host and act as a signal molecule for the production of nodulation factors by rhizobia. The perception of nodulation factors by the plant induces the root hair curling, facilitating the entry of bacteria in the cortical zone of plant tissue. Invaginations in the region of the hair bending, form the infection cord that leads the bacteria to the region where the nodule will be formed. The mitogenic action of nodulation factors stimulates the proliferation of cortical cells in the plant and the nodular primordium formation or emerging nodule. Rhizobia colonize the emerging nodule, forming the symbiosome, and differ in to bacteroids at the $N_2$ fixation stage

Nodulation factors act in several physiological processes in the host, in addition to those involved with nodulation, such as the formation of lateral roots [122]. Furthermore, LCOs activate the expression of genes involved in the plant cell cycle, stimulating cell division, not only in leguminous plants and, because of this, they stimulate germination, seedling growth and root growth in several non-target hosts, when applied in seeds [123,124]. They are also able to promote an increase in leaf area, increase in photosynthetic rate and total dry weight when are foliar inoculated [116]. Souleimanov et al. [125] observed that in submicromolar concentrations ($10^{-7}$ and $10^{-9}$ M) the purified LCO of *B. japonicum* 532C increased the germination rate of maize, rice, soybean, and promoted greater biomass accumulation, supporting the theory of the hormone-like action of these molecules proposed by Fisher and Long [126].

Some studies also report that exudates from non-leguminous plants act as inducers of the genes responsible (nod genes) for nodulation in rhizobia [116]. Lian et al. [127] observed the production of LCOs in a culture of *B. japonicum* after the addition of corn, soybean and wheat root extracts. The corn root

extract induced the production of LCOs by the bacteria in high concentrations. The response to exudates from non-leguminous plants by rhizobia suggests that, in addition to acting in symbiosis with leguminous plants, these bacteria act as PGPRs and that nodulation factors have hormone-like action, such as stimulation of formation of lateral roots, allowing greater assimilation of nutrients and water by the host [116].

In leguminous plants it was observed that the treatment with LCO extracted from *R. leguminosarum* bv. *viciae* GR09 in pea (*Pisum sativum*) and vetch (*Vicia villosa*) seeds increased germination, biomass and nodulation efficiency [124]. Similar results were obtained in *Medicago truncatula* treated with LCO of *S. meliloti* via seed [118].

Nodulation factors combined with the use of host-specific rhizobia are beneficial to leguminous growth. The purified LCOs, in the absence of rhizobia, are sufficient to induce the root hair curling, cell division and the formation of nodule-like structures [128]. López-Lara et al. [129] added purified LCO from *Rhizobium* sp. GRH2 in *Phaseolus* sp. and *Acacia* spp. and observed the formation and deformation of root hair.

Several studies have been carried out with the exogenous application of LCOs in legumes and an increase in the nodules number and nitrogen concentration in the leaf, as well as greater expansion of the root area are reported. [118,124]. In non-leguminous plants, tolerance to high temperature is reported [117]. The use of LCO with rhizobia increases symbiotic competitiveness and can benefit the recruitment of soil rhizobia to increase nodulation efficiency [124].

Studies carried out in Brazil showed that soybean inoculation with strains of *Bradyrhizobium* spp. added with secondary metabolites, containing LCOs, extracted from *B. diazoefficiens* USDA 110 increased grain yield by 4.8% compared to treatment inoculated only with the inoculant recommended for this crop [31]. In corn, the addition of *R. tropici* CIAT 899 metabolites to the *A. brasilense* inoculant was evaluated and there was an increase of 11.4% in productivity [31]. The application of *A. brasilense* enriched with *R. tropici* CIAT 899 LCOs showed an increase in corn productivity in five, out of a total of six, experiments conducted compared to the non-inoculated treatment [32].

There are still few companies that produce bioformulations containing secondary metabolites, such as LCOs. Among the products available, there are those that carry the purified molecule, which can be inoculated via foliar or seed and bioformulations composed of the molecule and the bacteria (rhizobia or PGPRs) recommended for the crop of interest [130]. Products that use this biotechnology are marketed by multinational companies and recommended for both leguminous plants such as soybeans, peanuts and alfalfa and for non-leguminous plants such as maize and wheat.

Since 2011, a product called Ratchet® (Monsanto) based on LCO, recommended for foliar use in soybean and corn, has been commercialized in the USA, which act by stimulating photosynthesis, sugar production, increasing plant growth and resulting in better harvest performance. In experiments conducted between 2008 and 2010, an increase of, approximately, 4 to 5 bags of grains per hectare in maize and 1 to 2 bags of soybean grains were evaluated with the inoculation of the commercial LCO via foliar, according to the manufacturer. Another product in the same line, TagTeam LCO® (NexusBioAg), commercialized in the USA, combines *R. leguminosarum* and purified LCO, and it is recommended for the cultivation of lentils (*Lens culinaris*) and peas (*Pisum sativum*). Also in the USA, the LCO Promoter Technology® (Novozymes), which carries purified nodulation factors is commercialized. The formulation called

Optimize ST® (NexusBioAg), commercialized in Canada, carries LCO with *B. japonicum* in a single product, ensuring increased nodule formation, nutrient absorption and increased harvest production. In addition to rhizobia, LCOs are commercialized in bioformulations with other microorganisms that promote plant growth such as *Penicillium bilaiae*, in a product called JumpStart LCO® (Novozymes) (Canada). *P. bilaiae* is a natural occurring fungus in several types of soils and has been studied for many years due to the high production of important compounds for phosphate solubilization. Despite its potential, the use of this technology in Brazil is still incipient. Further studies are needed on crop responses in the field, since work with these molecules is still very restricted to controlled conditions.

*3.1.2 Molecules involved in biofilm formation.*

Biofilms are structures formed by cellular aggregates coated with an array of extracellular polysaccharides (EPS), proteins and lipids. The EPS is the main structural component of biofilms, being responsible for the architecture, stability and organization of the cellular agglomerate in micro-colonies. The structural components of biofilm provide resistance to desiccation, passive absorption of nutrients from the adhesion region, act as a carbon source and resistance to biotic stress conditions (protection against antimicrobial compounds and toxins) and abiotic (changes in pH and variations in temperature) [131]. In the soil, the formation of bacterial biofilm ensures the protection of both pathogenic and non-pathogenic bacteria to elevated temperatures, nutritional and water limitations in microenvironments, in addition to enabling the adhesion of these cells to various surfaces, such as the rhizosphere [131–133] (Figure 3).

The molecules involved in biofilm formation, such as EPS, have elucidated effects on the structure of bacterial micro-colonies and it is currently known that host plants recognize these molecular patterns and exhibit physiological responses [134–137]. Although they have physiological effects on plants and are crucial for the colonization of rhizobia and other PGPRs, there are still no formulations available on the market that have bacteria-bacteria communication molecules in their composition. The use of this type of molecule in formulations has a promising potential in increasing the rhizobia colonization on the root surface and improving the inoculant colonization efficiency.

Quorum-sensing (QS) is defined as an intra and / or inter-specific mechanism for regulating the density of the microbial population, mediated by molecules called autoinducers that act in cell-cell communication. This communication system between bacteria acts in the coordination of bacteria cells and has already been reported for many species of Gram-negative bacteria [138]. The main self-inducing molecule produced by this group of bacteria is N-acyl homoserine lactone (AHL) [139]. They are a group of molecules with low molecular weight and diffusible by the cell membrane that act in the expression of specific genes in response to environmental changes [131,132,135,140,141]. Among the phenotypic responses to the action of the QS, the most studied and relevant are the genes expression involved in virulence, biofilm formation, production of exopolysaccharides (EPS), colonization and symbiosis [139,142–145].

The QS process in rhizobia is crucial for the surface polysaccharides production, adaptation to the stationary phase, the symbiosome development, nodulation efficiency, since it is associated with the biofilm and micro colonies formation, which are crucial steps for the bacterial colonization in the rhizospheric region [133]. Pérez-Montaño et al. [143] showed that in the presence of flavonoids, the biofilm structure of *S. fredii* SMH12 change from monolayer to micro colony and then the effective colonization of soybean

cv. Osumi, which is a crucial stage for the nodules formation. Pérez-Montaño et al. [134] observed that the total molecules production involved in the QS in *S. fredii* SMH12, *R. etli* ISP42 and *R. sullae* IS123 is dependent on the type of flavonoid that is used.

The production of AHL and consequently the biofilm formation in the rhizosphere is crucial for the colonization of several microorganisms, in addition to rhizobia. It is now known that plants recognize a wide variety of AHL-type molecules and these modify the root architecture. Ortíz-Castro et al. [137] evaluated the biological activity of AHL in root development, using molecules with an acyl chain ranging from 4 to 14 carbons and observed a reduction in the primary root, lateral root and root hair growth in *Arabidopsis thaliana*, especially when applied to the N-decanoyl-HL (C10-HL). This study suggests that plant growth-promoting bacteria produce molecules of type C8 to C12-HL, and these molecules are those that have the greatest biological activity in root development. These results coincide with studies of Pérez-Montaño et al. [134], that when evaluating the molecules produced by *S. fredii, R. sullae and R. etli*, identified that in all cases there was the production of C8-HL. These results suggest that rhizobia and other growth-promoting bacteria possibly have the same molecular pattern, which is recognized by the host plant and acts by stimulating root development as a strategy to reinforce interactions with the bacterial partner [137,145].

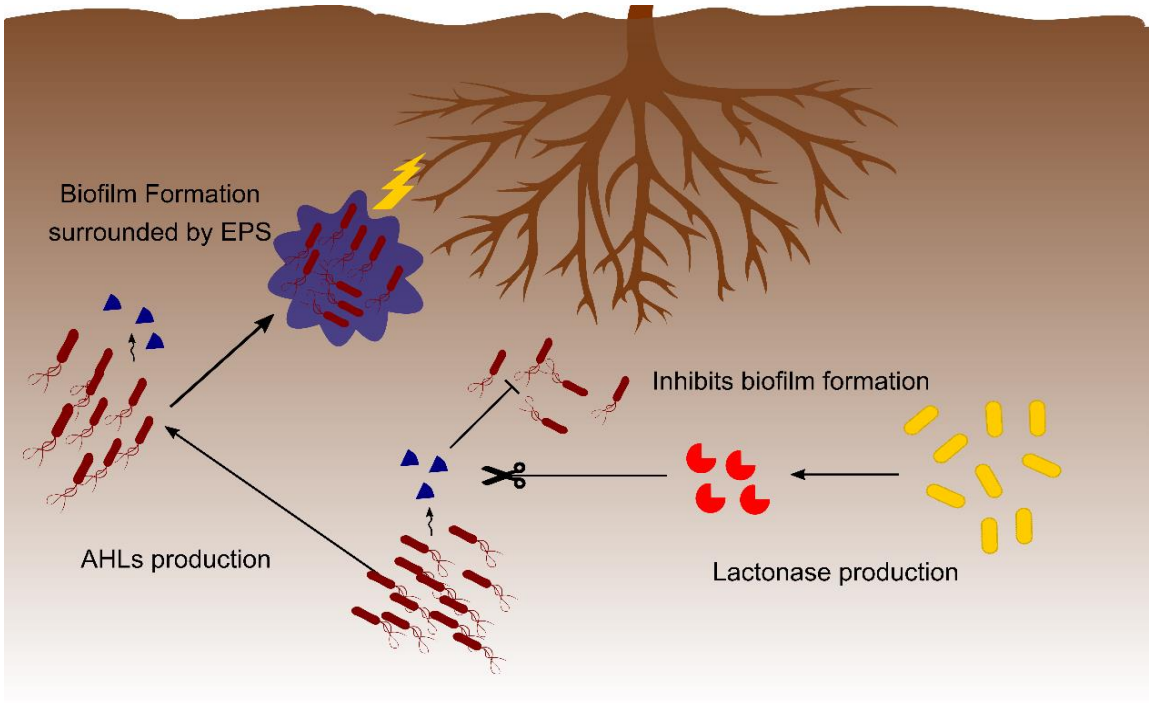


**Figure 3**. Biofilm formation by soil bacteria and action of lactonase enzymes. Soil bacteria control cell density by molecular signals called autoinducers. N-acyl homoserine lactone (AHL) is the main cell-cell communication molecule studied, and acts directly in the control of cell density and regulates the expression of genes involved in the biofilm formation (formed by exopolysaccharides - EPS) and virulence. Some bacterial genera, especially involved in biological control, produce enzymes called lactonases that act by breaking the lactone ring and preventing signaling via AHL. The lactonase enzymes action is related to the attenuation of the pathogenic bacteria virulence and the reduction of bacterial biofilms formation, both by pathogenic bacteria and plant growth promoters.

***3.2 Microbial carriers and bacteria bioencapsulation.***

The encapsulation, or immobilization, of microorganisms comprises an alternative technology that aims to protect the microbial cells in the soil and promote their gradual release [146–148]. The polymeric matrices can be composed of alginates, clay, agar, pectin, chitosan, polyacrylamide and gum, such as xanthan gum, which have different rates of degradation. Once encapsulated, cells are protected in a matrix permeable to water and nutrients that protects microorganisms from mechanical damage and environmental stress [149,150]. The slow and gradual degradation of the material releases the microorganisms continuously in the environment, allowing the inoculum to remain in the soil for a longer time [11,147,151–153]. The advantages associated with the use of PGPR encapsulation, according to conventional formulations, are in increasing the effectiveness of inoculants, in the controlled and gradual release of bacteria, in reducing the toxic effects of agrochemicals in seeds and in soils. These products are biodegradable and non-polluting and provide physical protection for the inoculum increasing its shelf life [11].

Among the matrices used, sodium alginate is the most common for agronomic uses [11,146,148]. The preparation of these matrices is done by adding the sodium alginate in the same solution as the inoculum, and through the addition of a calcium chloride solution, alginate particles are formed. These are washed and later lyophilized or prepared in liquid emulsions for stabilization in microcapsules [150,153].

Several methodologies are used to define particles size, shape and texture that will vary from the type of microorganism studied to the application method. Macroencapsulation are particles that vary in size from a few millimeters to centimeters but offer little contact with the seed. Microencapsulation, in turn, are particles of size ranging from 10-100 µm and offer greater contact between the inoculum and the seed [150]. The review by John et al. [153] details several technologies used in the production of these particles.

The PGPR encapsulation was first proposed by Bashan [154] and, in spite of all the aforementioned benefits, it still has no applications in the field and there is no large-scale production. One of the main reasons for this is the difficulty in maintaining a completely sterile and contaminant-free environment; cell mortality in the lyophilization stage, with a significant reduction in cell concentration; in addition to a production high cost superior to peat and liquid formulations [153,155]. In this sense, research has sought to circumvent these obstacles with the search for alternatives that reduce the bottlenecks that still exist for the diffusion of this technology. Kadmiri et al. [156] showed that the use of hybrid polymeric matrices composed of calcium alginate of two types of clay was efficient in preserving the concentration of *P. fluorescens* Ms-01 and *A. brasilense* DSM1690 (Ab) cells for three months at room temperature and when added to a saline solution, biocapsules released the inoculum for 15 days, revealing a slow and gradual release capacity. In addition, the authors found that the inoculation of these particles in wheat significantly increased the root and biomass area and the accumulation of nitrogen in the roots, regarding to the uninoculated control. Young et al. [157] observed that the viability of PGPR *B. subtilis* CC-pg 104 cells encapsulated in a matrix of sodium alginate and humic acid was not altered after lyophilization during five months storage at room temperature. This study also showed that the bacteria encapsulation increased the persistence of the bacteria by $10^4$ CFU (cm of root $^{-1}$) in the rhizosphere and by 10 times the number of CFU cm of root $^{-1}$ in the rhizoplane compared to free cell inoculation. On an industrial scale Strobel et al. [151] studied a spray drying method that combines the use of high temperatures and cell dehydration for the production of calcium alginate microparticles containing PGPR *Methylobacterium radiotolerans*. It was

observed that *M. radiotolerans* cells maintained their viability after the process in a concentration of $10^{10}$ CFU $g^{-1}$ of lyophilizate, but there was a decline in the bacterial population after one year of storage. The authors reinforce that the methodology used is applicable at an industrial level for the inoculants production for seeds or foliar application. These studies show that the use of bacterial encapsulation for agronomic applications is close to becoming a reality for producers. The encapsulation techniques improvement has allowed a reduction in production costs, as well as an increase in the microorganisms viability, which can bring significant promises both for use in microbial inoculants and biopesticides formulations.

***3.3 Seed pre-inoculation technology.***

Inoculants production involves, in addition to contaminant-free microbial growth, the use of a carrier formulation that should provide favorable conditions for maintaining the microorganism viability and cell concentration for as long as possible. The desirable characteristics in a carrier are: do not have toxic substances to the microorganism; be easily sterilizable; have an adequate and buffered pH; allow the microorganisms initial growth and ensure cell viability and concentration for as long as possible [158]. The choice of a suitable carrier is crucial for the production of a microbial inoculant, since any factor that acts by reducing the rhizobia cells concentration, consequently, reduces the BNF efficiency [159].

For decades, peat has been the main carrier used in several inoculants. This material is rich in organic matter, which acts as a nutritional reserve for microorganisms and protects cells from osmotic stress conditions. This carrier ensures that the product final formulation maintains cell concentration and viability and is free from contaminants, in accordance with the Brazilian legislation [159]. Peat inoculation should be performed using an adhesive agent that will allow the product to contact the seed, in Brazil, a 10% sucrose solution is commonly used [36,159]. However, inoculation with peat in large production areas consumes a long period at sowing and requires specialized machinery. In addition, peat is a non-renewable product in nature and can generate irreversible environmental impacts [158]. In this sense, new formulations are demanded by the market, being the most accepted currently the liquid formulation.

Liquid formulations are produced from the bacterial culture medium with stabilizing agents such as mineral and organic oils and cell protectors and their use is less laborious and, generally, with the same quality as peat formulations [11]. Alternatively, these formulations allow the use of new inoculation techniques such as the foliar inoculation or in furrow, which can be an advantage, as in cases of remedying the inoculation, if it has not been done correctly via seed or to avoid contact of the inoculant with agrochemicals treated seeds [24,158,160,161]. Due to its great usefulness, it currently comprises 80% of the total doses of inoculants sold in Brazil [44].

Despite advances both in the inoculant formulation and in the application techniques, there are still some important issues to be addressed. The low viability of the microorganism after inoculation in the seed makes it necessary that the inoculation occur within a period of up to 24 hours before sowing, both in the products use with peat carriers and liquids [160]. The short period since inoculation until sowing might reduce the process quality and delay the work in the field and it demands a larger number of people for the process. Furthermore, inoculation carried out in the planting area itself requires specialized machinery, which can be a decisive factor in adhering to the use of technology and if performed incorrectly reduces its efficiency [162].

Pre-sowing seed inoculation has emerged as an alternative technique to the practice of inoculation, which consists in the seeds previous inoculation that can occur days and even weeks before sowing [160]. The benefits associated with the pre-inoculation technique include a reduction in the possibility of inoculation errors, which can result in a poor microorganisms distribution in the seeds and, consequently, a reduction in the technique efficiency. The availability of pre-inoculated seeds reduces one of the steps in the sowing process and guarantees the efficiency of inoculation, these gains can increase the producers search for this technology [162].

In an experiment conducted in four different agricultural areas in Brazil, Hungria et al. [44] found that pre-inoculated seeds 15 days before planting did not show statistical differences in productivity and nitrogen accumulation in the grain compared to seeds inoculated at the time of planting. The authors show that the treatments average productivity with pre-inoculation was 89% higher than the non-inoculated and non-fertilized control, pointing out that the use of this technique guaranteed the efficiency of the BNF. However, the study was carried out on seeds not treated with agrochemicals. Much of the totality of seeds used in large plantations in Brazil are treated with fungicides and chemical additives that are toxic agents to microorganisms and can drastically reduce their population in the seed [163]. A long period of exposure of the inoculum with the chemical agent reduces the number of cells capable of forming nodules and decreases the efficiency of BNF [159,160,164]. Campo et al. [163] found that two hours after inoculation of *Bradyrhizobium* sp. in soybeans previously treated with fungicides, 62% of the initial cell population was no longer viable, and after 24 hours only 5% remained viable.

Alternatively, several studies have been conducted in search of cell protectors that are biopolymers that maintain optimal water activity for the rhizobia survival in the cell and reduce the contact of the bacteria with the seed pesticides [165]. Neto et al. [166] suggest that the use of additives as cell protectors is efficient in the pre-inoculation of *Bradyrhizobium* sp. in soybean up to 45 days before sowing. Sandini et al. [167] show that seeds pre-treated with insecticides and fungicides and inoculant added to a cell protector maintain cell viability without compromising BNF (estimated by the number of nodules and nitrogen in the aerial part of the plant) for more than 71 days in storage. According to Araujo et al. [161] seed pre-inoculation is feasible if associated with the use of cell protectors. According to the results found, the use of cell protectors increased nodulation, grain yield and plant development in seeds inoculated 30 days before planting compared to treated seeds inoculated at the time of sowing without the presence of cell protectors [161].

One of the first products in the Brazilian market for seeds pre-inoculation was the Biagro NGTM (Bayer). This product was developed in a partnership between Embrapa and Bayer, and since 2013 it has been available on the market. However, the manufacturer does not recommend it for using in seeds treated with pesticides and guarantees the cells viability for up to 15 days. In the Brazilian market, there are companies that already offer seeds pre-inoculation technology combined with the treatment with pesticides.

Bayer markets the CTS 500® and, according to the manufacturer, is compatible with the main nematicides, insecticides and fungicides used in soybean seeds, with cell viability up to 60 days. Granouro® (Basf) is a kit composed of *B. elkanii* SEMIA 587 and SEMIA 5019, an adhesive and a protective agent that is applied during industrial seed treatment and guarantees cell viability without compromising the nitrogen supply up to 45 days from seed application. In the 2019/2020 crop season, the Rizoliq LLI product, from Rizobacter, was used in more than one million hectares of soybean. This inoculant has an

osmoprotective agent that protects cells from *Bradyrhizobium* spp. SEMIA 5079 and SEMIA 5080 for up to 60 days, according to the manufacturer. All of these products are commercialized in Brazil.

**4.0 Conclusions and perspectives.**

For more than 50 years, inoculants containing rhizobia have been marketed in Brazil and, more recently, products containing other microorganisms have shown benefits and gained acceptance from farmers. Currently, bio-based products have been more studied as for the interest in a more sustainable agriculture and for the gains in productivity that these products offer. As reported in this review, we have a scenario of biotechnological innovations aimed at the development of new products or agricultural processes that search to facilitate the management of the farmer and ensure greater efficiency and, consequently, greater crop productivity. Therefore, a promising agricultural scenario is expected in the coming years and decades with the launch of biotechnological innovations.

We are currently living global emergencies such as agricultural areas desertification and climate change that can alter the entire methods of food production in the world. In that regard there is an urgent search for biological solutions that can mitigate the effects that these environmental impacts may have on food production. The search for biological solutions in agriculture is a matter of food security, and in this sense, new products and technologies must be developed and the use of microbiological products must be a reference in the Brazilian and global agricultural market in the coming years.

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# Chapter 2 - Secondary metabolites of *Rhizobium tropici* CIAT 899 added to *Bradyrhizobium* spp. inoculant promotes soybean growth and increases yield[1]

*"We became scientists because we are curious - we are driven to solve the puzzles that nature presents"*

*Joshua Schimel*


**Abstract**

Purpose: Products based on beneficial microorganisms, such as rhizobia, are used in agriculture to reduce or even completely replace the use of mineral fertilizers. In Brazil, rhizobia inoculants have been widely used in agriculture in the cultivation of legumes such as soybeans. Currently, the development of new formulations based on microorganisms have been made to improve the effectiveness of these products. Among these new technologies, the use of secondary bacterial metabolites has been studied to compose new formulations of inoculants. Inoculants enriched with metabolic extract from rhizobia have been showing promising results in plant growth promotion, but little is known about the isolated effects of the molecules contained in this extract, such as the lipochitoligosaccharides (LCOs), chitoligosaccharides (COs) and exopolysaccharides (EPS), when they are purified. Methods: In this study, one greenhouse and two field experiments were carried out to evaluate the effects of the addition of secondary metabolites from *Rhizobium tropici* CIAT 899 to the standard inoculation (SI) with *Bradyrhizobium* spp. on soybean nodulation, growth and yield. Total metabolic extract containing LCOs (ME-LCO), and other secondary metabolism molecules, such as purified LCOs, COs and EPS were tested. Results: In the greenhouse, it was observed that the addition of ME-LCO increased nodule number and shoot, root and nodules dry weight in comparison to the SI. The SI + ME-LCO treatment significantly increased soybean grain yield by 7.6% compared to SI in the 2018/2019 cropping season. In 2019/2020 an increase of 3.1% was observed, but it was not statistically significant. Conclusions: The use of ME-LCO of *R. tropici* CIAT 899 in supplementation of *Bradyrhizobium* spp. soybean inoculants is very promising for the formulation of new generation inoculants.



**Keywords:** Nod factors; biological nitrogen fixation; lipochitoligosaccharides, chitoligosaccharides; exopolysaccharides, sustainable agriculture


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**Introduction**

Microbial inoculants, also called biofertilizers, are formulations that contain microbial agents of agricultural interest, which promote plant growth (Adesemoye and Kloepper 2009; Baez-Rogelio et al. 2016). The use of this biotechnology has grown considerably in the last few decades as it is a sustainable alternative to substitute or reduce the use of mineral fertilizers (Singh et al. 2011; Callaghan 2016). The use of inoculants in agriculture is a consolidated practice in Brazil, especially in formulations composed of rhizobia. Currently, Brazil is the main producer and exporter of soybean (*Glycine max* [L.] Merr.) in the world and approximately 90% of its planted area is inoculated with elite strains of *Bradyrhizobium* spp. (Hungria et al. 2020).

Most of the microorganisms of agronomic interest can increase the availability of essential nutrients for plant growth. In return, the plant provides photoassimilates as carbon sources to the microbial agents (Oldroyd 2013). Rhizobia are soil bacteria capable of forming symbiotic interactions with leguminous plants and carrying out biological nitrogen fixation (BNF) in specialized organs, called nodules (Burian and Bensmihen 2018; del Cerro et al. 2019b). These symbiotic associations need communication between the host plant and the microorganism to occur. Initially, the host plant releases flavonoids molecules in the rhizosphere that are recognized by the transcriptional factor NodD, constitutively produced by rhizobia, and activates the expression of the nodulation genes (*nod* genes) (Hungria and Stacey 1997; Burian and Bensmihen 2018; del Cerro et al. 2019b).

Nodulation genes are involved in the biosynthesis of nodulation factors (NF) or lipochitoligosaccharides (LCOs). The LCOs have a chitooligosaccharide (CO) backbone composed of three to six residues of N-acetylglucosamine (GlcNAc) with β 1-4 linkages (Oldroyd 2013; del Cerro et al. 2019a). The specificity of the LCOs molecules occurs with the addition of substituent groups to the CO chain such as methyl, fucosyl, acetyl and sulfated groups. Another determining factor for the formation of the LCOs is the degree of saturation in the acetyl groups (Liang et al. 2014). The decorations, chain size and degree of saturation are produced differently among the rhizobia and their composition is decisive for the specificity of the leguminous-rhizobia communication (Lian et al. 2002; Souleimanov et al. 2002; Oldroyd 2013).

LCOs are key molecules for the occurrence of the symbiosis. When recognized by the plant host, the LCOs trigger various physiological responses, such as the formation of nodular primordia, cell division and the formation of the infection thread that allows the rhizobia to enter into the roots (Dénarié and Cullimore 1993; Dénarié et al. 1996; Kannenberg and Carlson 2005). After infection of the rhizobia and the formation of a mature nodule, the BNF process begins (Souleimanov et al. 2002; Liang et al. 2014; Burian and Bensmihen 2018). When applied exogenously, the LCOs alter root architecture, stimulate germination and promote plant growth, similarly to plant growth regulators, such as auxin (Souleimanov et al. 2002). In addition, LCOs stimulate an increase in the number of nodules and accumulation of nitrogen when applied to leguminous plants (Macchiavelli and Brelles-Mariño 2004).

A recent work carried out in Brazil has shown that soybean inoculation with *Bradyrhizobium* spp. strains supplemented with secondary metabolites extracted from *B. diazoefficiens* USDA 110 increased grain yield by 4.8% compared to standard inoculation (Marks et al. 2013). Because of the beneficial effects in plant growth, the use of LCOs in inoculant formulations has gained focus in research and is part of a new generation of inoculants (Marks et al. 2013, 2015; Figueiredo et al. 2016; Schwinghamer et al. 2016; Moretti et al. 2020a, 2021).

The COs, also present in the exoskeleton of arthropods, as well as in the cell wall of filamentous fungi and yeast, are often associated with the development of immune responses in plants (Khan et al. 2011). Similarly to LCOs, the use of exogenous COs is related to the stimulation of physiological responses in the host plant. Khan et al. (2011) observed that *Arabdopsis thaliana* treated with submicromolar concentrations of a CO had 26% longer roots than those that did not receive the CO treatment. Oláh et al. (2005) observed that the use of a CO in *Medicago truncatula* stimulates the formation of lateral roots and is also associated with greater stimulation of mycorrhizal formations. However, there are still few studies that assess the exogenous use of COs in promoting plant growth.

Another group of bacterial molecules that present interesting effects on plants are the exopolysaccharides (EPS). These molecules are necessary for the nodulation process and nodular occupation (Staudt et al. 2012; Ghosh and Maiti 2016). The EPS have an important role in the biofilm formation and increasing the bacterial adherence to root hairs (Castellane et al. 2015).

The use of LCOs and other molecules of rhizobia metabolism stand out as a promising strategy to compose a more sustainable and productive agriculture. Although it is well known that inoculants enriched with secondary metabolism molecules of rhizobia are promising alternatives for the formulation of more effective bioproducts (Prithiviraj et al. 2002; Marks et al. 2013, 2015; Schwinghamer et al. 2015; Barbosa et al. 2020; Moretti et al. 2020a), the application of this type of formulation in plants of agricultural interest is still very incipient. *Rhizobium tropici* CIAT 899 is currently recommended for common bean inoculation in Brazil and this strain produces a wide variety of LCOs molecules (Ormeño-Orrillo et al. 2012; Guasch-Vidal et al. 2013). In previous studies, the addition of total metabolic extract of *R. tropici* CIAT 899 induced by flavonoid to inoculants for maize and soybean promoted growth and increased productivity compared to standard inoculation (Marks et al. 2015). On the other hand, there is very little information about the isolated effects of the molecules contained in this total metabolic extract, such as LCOs, CO and EPS, when they are purified.

In the present study, a total metabolic extract containing LCOs (ME-LCO) and other purified secondary metabolites, such as LCOs, CO and EPS, extracted from *R. tropici* CIAT 899, were added to the commercial *Bradyrhizobium* spp. inoculant recommended for the soybean crop. We hypothesize that the combination of the standard inoculant, which has solid field results and wide commercial acceptance, supplemented with molecules from the secondary metabolism of *R. tropici* CIAT 899, important in rhizobia-legume communication, will act to promote plant growth and increase soybean yield. To test this hypothesis, one greenhouse and two field experiments were carried out for better understanding and evaluation of the effects of the combination of the inoculant enriched with these molecules on soybean nodulation, growth, and grain yield.

## Materials and Methods

### *Microbial inoculants*

Commercial liquid inoculants formulated with the strains *Bradyrhizobium japonicum* SEMIA 5079 (= CPAC 15) and *B. diazoefficiens* SEMIA 5080 (= CPAC 7) were used. The combination of these two strains is commercialized in Brazil and guarantees the supply of the nitrogen demanded by the soybean crop (Hungria et al. 2006).

*Extraction and purification of secondary metabolites from Rhizobium tropici* CIAT 899

The extraction of secondary metabolites involved with the nodulation of *R. tropici* CIAT 899 (= SEMIA 4077) was carried out according to Marks et al. (2013, 2015). The *R. tropici* CIAT 899 was grown in 1l of minimal $B^-$ medium supplemented with the inducing flavonoid apigenin, in a concentration of 1.0 µl $ml^{-1}$, and stirring at 180 rpm at 28 °C. After 48 hours, 333 ml of butanol was added and the formation of two phases was observed. The organic phase was maintained in a rotary evaporator and the crude extract was eluted in a 20% acetonitrile solution to obtain the total metabolic extract (ME-LCO). The purification of lipoquitooligosaccharides (LCOp) and chitooligosaccharide (COp) was carried out from the aqueous phase of the butanolic extract, in reversed phase thin layer chromatography (RP-TLC), with SPE C18, according to the protocol described by Guasch-Vidal et al. (2013). EPS was extracted according to the Staudt et al.(2012) protocol using mannitol (1%) as a carbon source and ethanol as the precipitating agent. The *R. tropici* CIAT 899 is deposited in the Culture Collection of Multifunctional Organisms of Embrapa Cerrados (Brasília, Federal District - Brazil).

*Biological activity of CO*

COp was used for analysis of biological activity evaluating the formation of nodular primordia. The soybean seeds were sterilized in 96% alcohol for 30 seconds, followed by 5 minutes in 3% sodium hypochlorite and washing with sterile water (Vicent 1970). These seeds were pre-germinated for 72 hours in agar-water medium and the seedling was deposited in tubes containing 30 ml of Farhaeus solution (Fahraeus 1957) supplemented with 0.2; 2.0 and 20 µl $ml^{-1}$ of COp or approximately $10^{-9}$, $10^{-8}$ e $10^{-7}$ M COp, respectively. A control without the addition of COp was also used. The experiment was carried out using six tubes, with one seedling each, per treatment. The plants were grown for ten days in a growth chamber with 16 hours of light at 26 °C and 8 hours in the dark at 18 °C and with constant humidity equal to 70%. The presence of nodular primordia was evaluated by bleaching the roots submerging them in sodium hypochlorite 10% followed by staining with methylene blue (Truchet et al. 1989).

*Greenhouse experiment*

The experiment was carried out in a greenhouse using Leonard jars with sterile substrate containing a mixture of sand and pulverized charcoal (1:1, v/v) with applications of Norris nutrient solution free of N when necessary (Norris and Mannetje 1964). Soybean seeds from the cultivar Brasmax Desafio 8473 RR were disinfected as previously described. The jars were grouped in a completely randomized block design with five replicates. Six treatments were evaluated: (i) control without nitrogen fertilization and without inoculation with *Bradyrhizobium* spp.; (ii) Standard inoculation (SI) with *Bradyrhizobium* spp.; (iii) SI + 1.0 µl $ml^{-1}$ ME-LCO; (iv) SI + 0.5 µl $ml^{-1}$ LCOp; (v) SI + 2.0 µl $ml^{-1}$ COp and (vi) SI + 62.5 µl $ml^{-1}$ EPS. Inoculation was carried out on seeds one hour before sowing to supply approximately $1.2 \times 10^6$ cells of *Bradyrhizobium* spp. per seed, as recommended for soybean in Brazil. Secondary metabolism molecules of *R. tropici* CIAT 899 were diluted in the inoculant before application to the seeds according to each treatment. Six soybean seeds were planted in each pot and the seedlings were thinned to two plants three days after complete germination. The plants were harvested 40 days after emergence (DAE). Shoots were detached and the roots were washed and dried at room temperature for two hours before the nodules were removed and

counted. After that, shoot, root, and nodules were dried at 60 °C for 72 hours and then weighed. The total N concentration in shoots (TNS) was determined by the modified Berthelot perchloric digestion method (Woolley et al. 1960).

*Field experiments*

*Site Description*

Two field experiments were carried out in neighboring areas in two seasons 2018/2019 and 2019/2020 in the experimental field of the Cerrados Agricultural Research Center (Embrapa Cerrados), Planaltina, Distrito Federal, Brazil (15º 35' 30'' S e 47º 42'30'' W, 1.175m above sea level). According to the Köppen-Geiger classification, the climate of the region is Cwa, typical savanna climate, characterized by rainy summer from October to April and a dry winter between the months of May to September (Lopes et al. 2013). The maximum yearly average temperature is 26.4ºC and the minimum 15.9ºC (Lopes et al. 2013). In both sites the soil is characterized as a typical Acrustox with clay texture (513 g $kg^{-1}$ clay; 186 g $kg^{-1}$ silt; and 301 g $kg^{-1}$ sand). Climatological data in the experimental periods and soil chemical properties at each site before planting are shown in Figure 4 and Table 1, respectively. Prior to sowing fertilization was performed with 60 kg $ha^{-1}$ of $P_2O_5$ and 60 kg $ha^{-1}$ of $K_2O$.

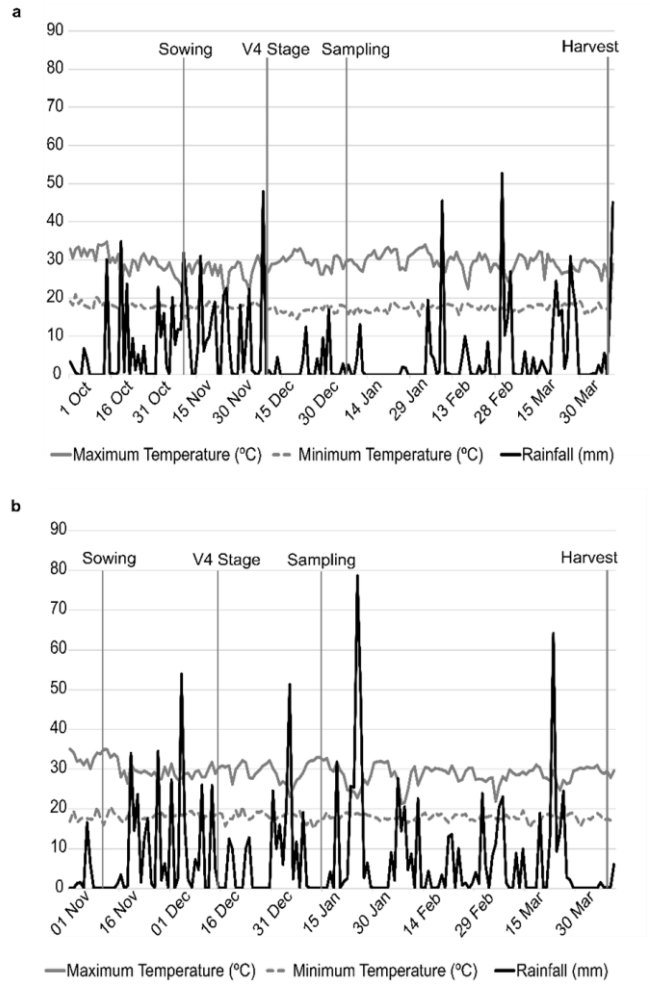


**Figure 4.** Rainfall and maximum and minimum temperatures in the experimental area of Embrapa Cerrados during the period of the experiments (a) Cropping season 2018/2019 (b) Cropping season 2019/2020.

**Table 1** Chemical characteristics of the soils in the experimental area of the Cerrados Agricultural Research Center (CPAC). Soil samples were taken from the 0-20 cm layer and all analyses were performed before sowing.

| Crop season | pH | Al | Ca | Mg | H+Al | K | P | OM | V |
|---|---|---|---|---|---|---|---|---|---|
| | | | ---------- $cmol_c.dm^{-3}$ ---------- | | | -------- $ml.L^{-1}$ -------- | | ------- % ------- | |
| 2018/2019 | 5.99 (0.060) | 0.035 (0.005) | 2.15 (0.053) | 0.84 (0.029) | 2.74 (0.094) | 74 (11.72) | 11.01 (0.834) | 1.66 (0.082) | 53.83 (1.337) |
| 2019/2020 | 5.51 (0.064) | 0.121 (0.025) | 1.67 (0.117) | 0.74 (0.075) | 5.38 (0.179) | 144.16 (5.230) | 18.14 (2.293) | 2.54 (0.052) | 34.08 (2.359) |

Values are means of eight replicates (standard error in parentheses). OM; soil organic matter. V; bases saturation = [(K + Ca + Mg)/CEC] × 100. CEC; cation exchange capacity (SB + H + Al).

*Experimental design and treatments*

The experimental design was a completely randomized block with six replicates. Each experimental plot measured 4 m (width) x 5 m (length), with 20 $m^2$ of planted area, with eight rows and 0.5 m spacing between the rows. A total of 100 seeds were planted in each row and the plots were 1.0 m apart from each other to avoid cross contamination.

The treatments were: (i) control without nitrogen fertilization and without inoculation with *Bradyrhizobium* spp.; (ii) control with nitrogen fertilization corresponding to 200 kg of N $ha^{-1}$; (iii) recommended standard inoculation (SI) for soybean culture in Brazil with *Bradyrhizobium* spp.; (iv) SI + 1.0 µl $ml^{-1}$ ME-LCO; (v) SI + 0.5 µl $ml^{-1}$ LCOp; (vi) SI + of 2.0 µl $ml^{-1}$ COp and (vii) SI + of 62.5 µl $ml^{-1}$ EPS. *Bradyrhizobium* spp. and secondary metabolism molecules inoculation was performed as described for the greenhouse experiment. Nitrogen fertilization was performed, using urea, splitted 50% at 5 DAE and 50% at 50 DAE, only in the treatment with nitrogen fertilization. In the season 2019/2020 the treatment (v) SI + 0.5 µl $ml^{-1}$ LCOp was not evaluated.

*Plant sampling, harvesting and analyses*

At V4 growth stage (Fehr and Caviness 1977), approximately 30 DAE, six plants were collected, three from the second row and three from the seventh row from each plot. The plants were used to determine the shoot dry weight (SDW), number (NN) and dry weight (NDW) of nodules, and total N concentration in shoots (TNS). The analyses were performed as previously described. When physiological maturity was reached, grain yield and the total N concentration in grains (TNG) were evaluated. Yield (kg $ha^{-1}$) was assessed from the 8 $m^2$ central area of each plot and calculated for 1 ha with moisture of the grains corrected to 13%. The TNG was determined by the modified Berthelot perchloric digestion method (Woolley et al. 1960).

*Statistical analyses*

All analyses were performed using software R version 3.6.1. The results obtained were first subjected to normality and homogeneity tests. Subsequently, the variables were subjected to the analysis of variance (ANOVA). The variables of the greenhouse and field experiments that showed significant differences in the F-test were submitted to Duncan's post hoc analysis at $p \leq 0,05$. Statistical analyses were performed with the package *ExpDes.pt*. All boxplot graphics were prepared using the package *ggplot2*.

**Results**

*Biological activity of CO*

Interestingly, the increased concentration of the COp molecule directly and significantly induced the formation of nodular primordia. A three-fold increase in the number of nodular primordia was observed in the roots treated with 20 µl $ml^{-1}$ of COp when compared to that observed in plants treated with 0.2 µl $ml^{-1}$ (Figure 5). This data indicates that this molecule is biologically active.

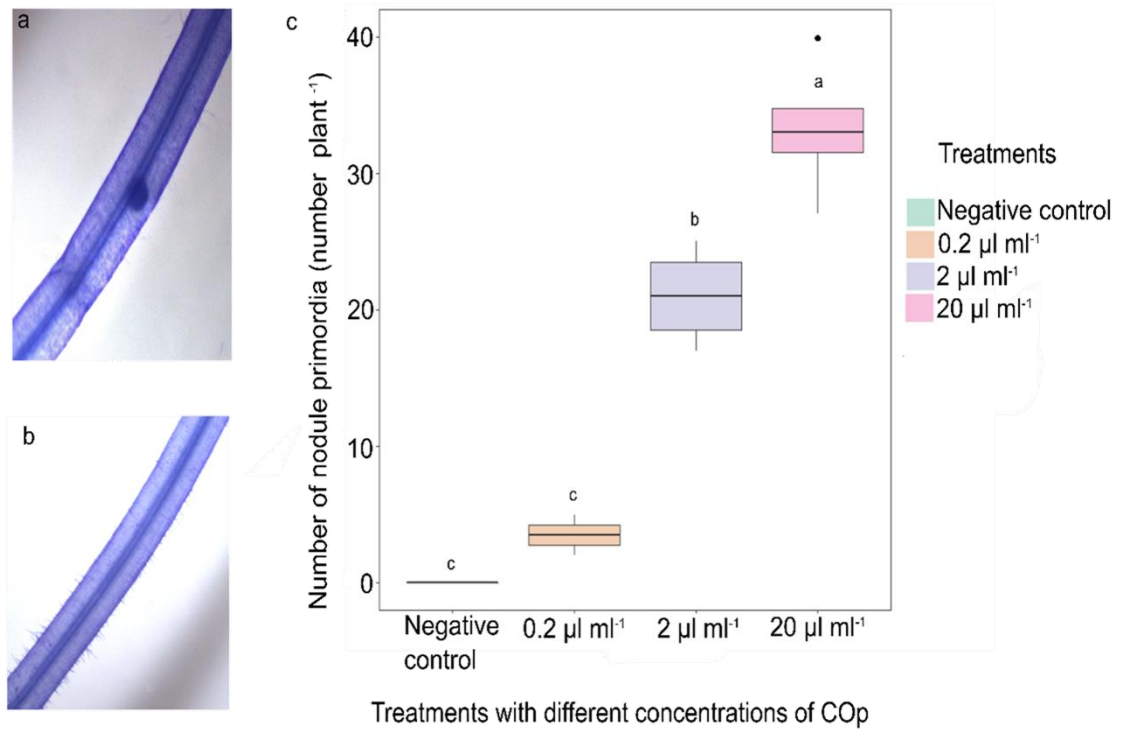


**Figure 5.** Nodular primordia in soybean cultivar Brasmax Desafio 8473 RR treated with 0.2 µl $ml^{-1}$, 2.0 µl $ml^{-1}$ and 20 µl $ml^{-1}$ of purified chitooligosaccharides (COp) extracted from *Rhizobium tropici* CIAT 899. (A) Nodular primordium in soybean treated with 20 µl COp $ml^{-1}$. (B) Negative control. (C) Boxplot of the number of nodular primordia in soybean treated with 0.2 µl $ml^{-1}$, 2.0 µl $ml^{-1}$ and 20 µl $ml^{-1}$ of COp extracted from *R. tropici* CIAT 899. Lower and upper box boundaries represent 25th and 75th percentiles, respectively; the line inside the box indicates

*Greenhouse experiment*

As showed in Table 2 all treatments were superior to the uninoculated control for all parameters evaluated. In four out of five parameters evaluated, SI + ME-LCO was the treatment that showed higher increases compared with SI, despite the non statistical significance, except for NDW. There was an increase of 18.5% in SDW, 8.4% in RDW, 15.2% in NN and a statistically significant difference of 23.5% in NDW. SI + ME-LCO also significantly incresead 41.75% the SDW in comparison to SI + EPS; 28% and 33.3% the RDW in comparison to SI + COp and SI + EPS, respectively; 22.3% the NN in comparison to SI + LCOp; and 29.2%, 25.1% and 31% the NDW, in comparison to SI + LCOp, SI + COp and SI + EPS, respectively. There were no differences between the inoculated treatments for TNS (Table 2).

**Table 2.** Shoot dry weight (SDW), root dry weight (RDW), nodule number (NN), nodule dry weight (NDW) and total N concentration in shoots (TNS) of soybean cultivar Brasmax Desafio 8473 RR at 40 days after emergence, in response to the standard inoculation (SI) with *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides - EPS). Experiment carried out under greenhouse conditions.

| **Treatments** | **SDW** (g plant$^{-1}$) | **RDW** (g plant$^{-1}$) | **NN** (plant$^{-1}$) | **NDW** (g plant$^{-1}$) | **TNS** (mg g$^{-1}$) |
|---|---|---|---|---|---|
| Non-inoculated control | 0.85 (0.02) $^{c}$ | 0.42 (0.01) $^{c}$ | 0 (0.00) $^{c}$ | 0 (0.00) $^{c}$ | 8.46 (0.93) $^{b}$ |
| Standard inoculation (SI) | 2.32 (0.42) $^{ab}$ | 0.59 (0.08) $^{ab}$ | 43.2 (3.01) $^{ab}$ | 0.318 (0.05) $^{b}$ | 34.57 (0.46) $^{a}$ |
| SI + ME-LCO | 2.75 (0.08) $^{a}$ | 0.64 (0.02) $^{a}$ | 49.8 (5.11) $^{a}$ | 0.393 (0.01) $^{a}$ | 34.29 (1.31) $^{a}$ |
| SI + LCOp | 2.20 (0.31) $^{ab}$ | 0.54 (0.06) $^{abc}$ | 40.7 (2.83) $^{b}$ | 0.304 (0.03) $^{b}$ | 36.97 (1.10) $^{a}$ |
| SI + COp | 2.18 (0.36) $^{ab}$ | 0.50 (0.05) $^{bc}$ | 43.7 (3.62) $^{ab}$ | 0.314 (0.05) $^{b}$ | 35.41 (1.35) $^{a}$ |
| SI + EPS | 1.94 (0.14) $^{b}$ | 0.48 (0.01) $^{bc}$ | 46.2 (2.83) $^{ab}$ | 0.300 (0.02) $^{b}$ | 26.56 (0.54) $^{a}$ |
| CV (%) | 25.83 % | 20.22 % | 17.78 % | 24.82 % | 6.98% |

Means (standard error in parentheses) followed by the same letter on the same column are not significantly different according to Duncan's test ($p \leq 0.05$).
CV = coefficient of variation.

*Field experiments*

In the experiment conducted in the 2018/2019 crop season, no significant differences in the NN were observed between the treatments. However, in the experiment conducted in 2019/2020, the NN in the treatment SI + EPS was higher than any other treatment, with an increase of 31.9% in comparison to the SI. NDW was significantly lower in the treatment that received N fertilizer when compared to the other treatments in both 2018/2019 and 2019/2020 cropping seasons. Also, in the 2019/2020 season, the NDW of the SI + EPS treatment was 25% higher than that of the SI. In the 2018/2019 experiment, in comparison to other treatments, SDW was significantly higher when plants received N fertilization, with an increase of 37.7% in relation to SI . In 2019/2020, there was no statistical differences in SDW between treatments. In 2018/2019, plants that were N fertilized showed the highest TNS, with an increase of 11.1% and in relation to SI. In 2018/2019, SI + COp was also higher than the SI for this parameter, with a 4.3% increase. In both crop seasons there were no differences between the treatments for TNG (Table 3).

**Table 3**. Nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW) and total N concentration in shoots (TNS) at the V4 growth stage and total N concentration in grains (TNG) at the physiological maturity of the soybean cultivar Brasmax Desafio 8473 RR in two experiments (2018/2019 and 2019/2020 crop seasons) located in the experimental field of the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides - EPS).

| Treatments | 2018/2019 | | | | | 2019/2020 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | V4 | | | | Harvest | V4 | | | | Harvest |
| | NN | NDW (g) per plant | SDW (g) per plant | TNS mg g$^{-1}$ | TNG mg g$^{-1}$ | NN | NDW (g) per plant | SDW (g) per plant | TNS mg g$^{-1}$ | TNG mg g$^{-1}$ |
| Control without N | 33.52 (2.71) | 0.12 (0.012) $^{a}$ | 1.46 (0.05) $^{b}$ | 38.96 (0.27) $^{c}$ | 53.48 (0.20) | 34.40 (1.53) $^{b}$ | 0.22 (0.013) $^{ab}$ | 2.52 (0.19) | 26.65 (0.37) $^{b}$ | 49.56 (0.72) |
| N fertilizer (200 kg ha$^{-1}$) | 31.55 (0.99) | 0.06 (0.005) $^{b}$ | 2.08 (0.04) $^{a}$ | 43.13 (0.78) $^{a}$ | 53.06 (1.12) | 30.40 (2.21) $^{b}$ | 0.09 (0.009) $^{c}$ | 2.25 (0.23) | 29.35 (0.64) $^{a}$ | 49.67 (0.60) |
| Standard Inoculation (SI) | 31.88 (1.20) | 0.13 (0.008) $^{a}$ | 1.51 (0.07) $^{b}$ | 38.82 (0.62) $^{c}$ | 52.47 (1.29) | 32.83 (3.04) $^{b}$ | 0.20 (0.028) $^{b}$ | 2.34 (0.06) | 29.13 (1.15) $^{a}$ | 49.19 (0.53) |
| SI + ME-LCO | 31.55 (2.19) | 0.11 (0.012) $^{a}$ | 1.50 (0.07) $^{b}$ | 40.16 (0.57) $^{bc}$ | 52.74 (0.88) | 36.70 (2.36) $^{b}$ | 0.21 (0.008) $^{ab}$ | 2.13 (0.12) | 25.77 (0.77) $^{b}$ | 49.22 (0.50) |
| SI + LCOp | 31.19 (1.20) | 0.12 (0.007) $^{a}$ | 1.46 (0.06) $^{b}$ | 39.89 (0.34) $^{bc}$ | 53.62 (0.82) | --- | --- | --- | --- | --- |
| SI + COp | 30.61 (2.61) | 0.12 (0.010) $^{a}$ | 1.49 (0.14) $^{b}$ | 40.49 (0.37) $^{b}$ | 52.45 (1.19) | 33.46 (3.15) $^{b}$ | 0.22 (0.016) $^{ab}$ | 2.27 (0.17) | 26.28 (1.39) $^{b}$ | 48.45 (0.87) |
| SI + EPS | 30.83 (3.48) | 0.11 (0.014) $^{a}$ | 1.46 (0.09) $^{b}$ | 40.23 (0.62) $^{bc}$ | 52.80 (0.59) | 43.26 (2.05) $^{a}$ | 0.25 (0.012) $^{a}$ | 2.45 (0.12) | 26.39 (0.23) $^{b}$ | 49.58 (0.39) |
| CV (%) | 16.5% $^{ns}$ | 17.8% | 12.1% | 2.78% | 3.93% $^{ns}$ | 14.6% | 15.9% | 15.7% $^{ns}$ | 7.78% | 3.19% $^{ns}$ |

Means (standard error in parentheses) followed by the same letter on the same column are not significantly different according to Duncan's test ($p \leq 0.05$).
CV = coefficient of variation.
ns = not significant ($P > 0.05$).
In the season 2019/2020 the treatment SI + 0.5 µl ml$^{-1}$ LCOp was not evaluated.

The addition of the ME-LCO to the inoculant based on *Bradyrhizobium* spp. increased the grain yield by 7.6% in relation to SI and was the only treatment that showed no statistical difference to the N fertilized treatment in the 2018/2019 crop season (Fig 3). This result reflects an increase of 370.8 kg of grains $ha^{-1}$ in relation to the treatment with just the SI. The other treatments with addition of molecules were statistically equal to SI and together with the ME-LCO, the SI + LCOp also showed higher yield than the uninoculated control. In 2019/2020, although the SI + ME-LCO treatment did not show a statistical difference to the SI in grain yield, an increase of 153.9 kg $ha^{-1}$ or 3.1% was observed. Also in this crop season, the SI + ME-LCO was the only treatment to differ from the uninoculated control, with an increase of 4.8% in grain yield (Figure 6). The N fertilized treatment presented the highest yield among all other treatments in 2019/2020 crop season.

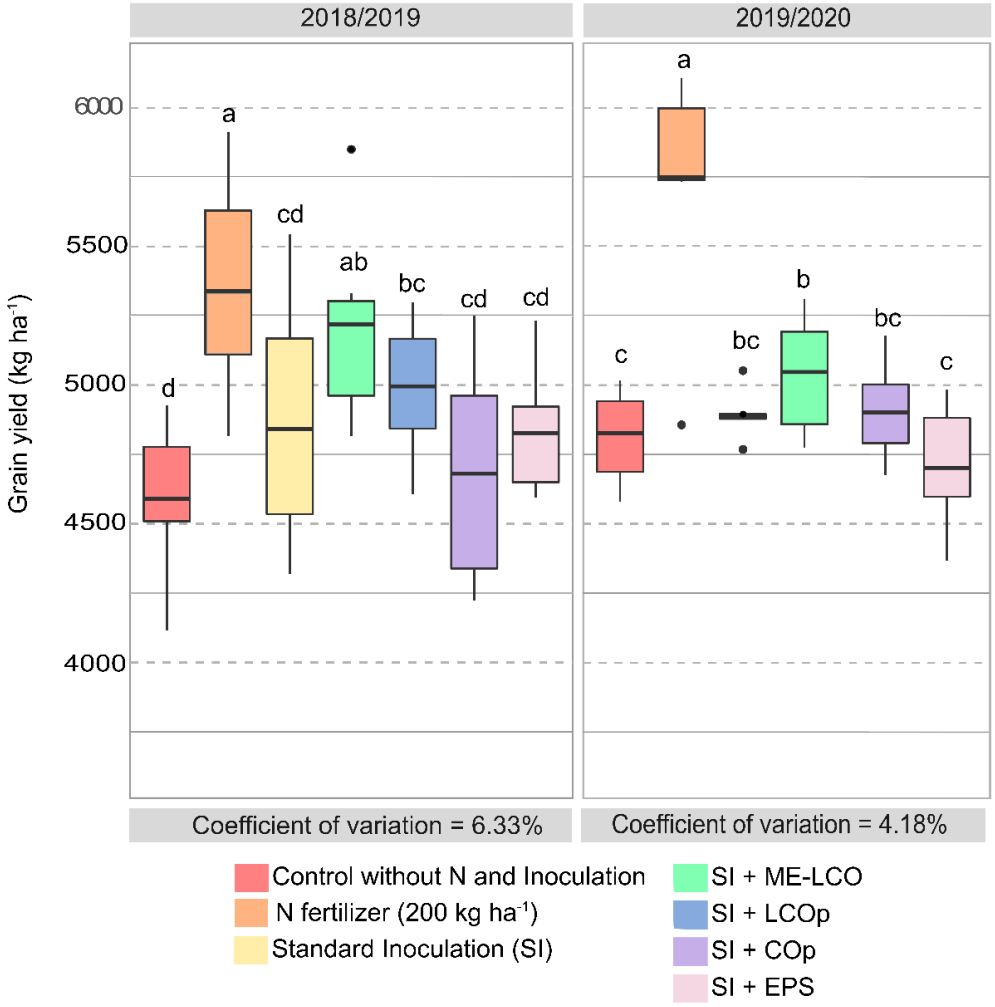


**Figure 6.** Boxplot of grain yield (kg $ha^{-1}$) of soybean cultivar Brasmax Desafio 8473 RR in two experiments (2018/2019 and 2019/2020 crop seasons) located in the experimental field of the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides - EPS). Lower and upper box boundaries represent 25th and 75th percentiles, respectively; the line inside the box indicates the median; whiskers on the top and bottom represent the 10th and 90th percentiles, respectively; points above and below the whiskers indicate outliers outside the 10th and 90th percentiles, respectively. Means followed by the same letter are not significantly different according to Duncan's test ($p \leq 0.05$).

The similarity dendrogram grouped the treatments into three main clusters according to the grain yield of the two field experiments. The control without inoculation nor N, SI + COp, SI + EPS and SI were grouped in a single cluster. The control and SI + COp treatments were more similar to each other, while the same was observed between the SI and SI + EPS treatments. The SI + ME-LCO and the N fertilizer treatments were isolated in a second and third clusters, respectively (Figure 7). With higher yields, it is interesting to highlight that, according to the dendogram, the SI + ME-LCO differed from other treatments with SI or SI + other secondary metabolism molecules and the N fertilizer treatments differed from all of them.

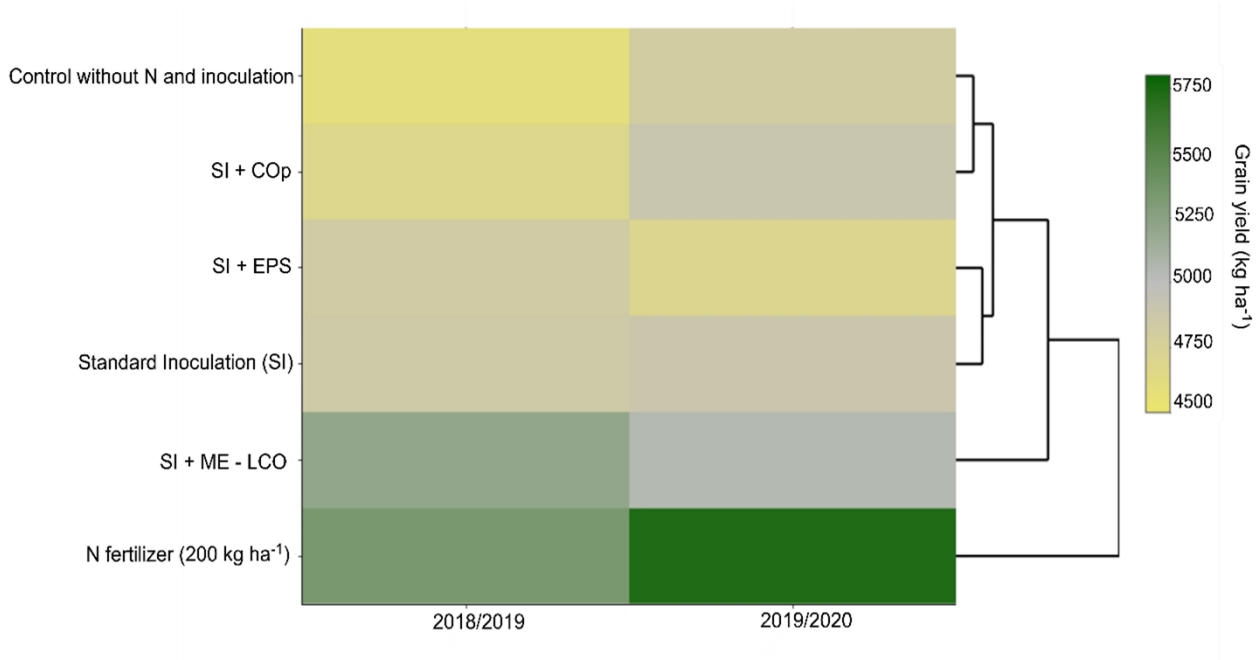


**Figure 7.** Heat map and dendrogram of the Pearson's correlation matrix for the grain yield (kg $ha^{-1}$) of the soybean cultivar Brasmax Desafio 8473 RR in two experiments (2018/2019 and 2019/2020 crop seasons) located in the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides - EPS). SI + LCOp treatment was not used in this analysis because it was not present in the 2019/2020 experiment.

**Discussion**

Sustainable agricultural practices have been the focus of several studies for many years as an alternative to the use of mineral fertilization. Brazil is currently the largest producer of soybean in the world, and the inoculation with *Bradyrhizobium* spp. is used to substitute N fertilization, resulting in annual savings of US $15 billion (Hungria et al. 2020). Approximately 70 million doses of inoculants containing elite strains are currently commercialized in Brazil every year (Hungria et al. 2020). However, agricultural sciences constantly seek to advance in technologies that combine productivity gains with sustainable practices. The mechanism of action of the secondary metabolism molecules of rhizobia, that have crucial role in communication with their hosts, have been deeply elucidated. It is well known, for example, that the nod factors are determinant for the formation of the nodules and to guarantee the maximum BNF (Brechenmacher et al. 2010; Schwinghamer et al. 2015; Smith et al. 2015). Due to this, formulations containing these

molecules have already been analyzed (Marks et al. 2013, 2015; Moretti et al. 2020a) but, there is still the need for practical studies that evaluate the action of these molecules in the field.

*R. tropici* CIAT 899 is recommended for common bean inoculation and previous studies demonstrated that this strain has the ability to survive in adverse environmental conditions such as acidic soils and produces a wide variety of LCO molecules, even in the absence of inducing flavonoids (Martínez-Romero et al. 1991; Ormeño-Orrillo et al. 2012; del Cerro et al. 2017). These characteristics make it a suitable and interesting strain as a source of secondary metabolites. Although the response of soybean inoculated with standard inoculant plus the total metabolic extract (ME-LCO) of *R. tropici* CIAT 899 has been reported (Marks et al. 2013) it has not yet been shown if different fractions of the metabolic extract could promote the growth of soybean.

Firstly, to determine the biological activity of the chitooligosaccharide (COp), we analyzed the effect of different concentrations of this molecule on the soybean root. Although it is known that LCOs act in the organogenesis of nodules in submicromolar concentrations (Dénarié and Cullimore 1993; Dénarié et al. 1996) and that *R. tropici* CIAT 899, in the presence of apigenin, synthesizes LCOs that induce the formation of nodular primordia (Pérez-Montaño et al. 2016), the contribution of CO to nodular formation is still unclear. The results obtained in this study show that the concentration of COp in the nutrient solution significantly affected the formation of nodular primordia in soybean roots (Figure 5). A three-fold increase in the number of nodular primordia was observed in the roots treated with 20 μl $ml^{-1}$ of COp when compared to that observed in plants treated with 0.2 μl $ml^{-1}$. These results confirm that CO have biological activity and are consistent with previous studies that reported that CO composed of four to five residues of N-acetylglucosamine can induce an increase in intracellular calcium concentration, similar to nodulation factors, an important process for nodulation signaling (Walker et al. 2000). Moreover, another study showed that chitin pentamers induce the transcriptional activation of genes associated with nodulation in soybean (Minami et al. 1996). The results found reinforce the hypothesis of this work that proposes that the use of secondary metabolism molecules of *R. tropici* CIAT 899, being COp one of these molecules, can benefit and increase the performance of microbial based products in the field.

In this study, we also investigated the agronomic effects of supplementing inoculants composed of *Bradyrhizobium* spp. with molecules of secondary metabolism of *R. tropici* CIAT 899. In the greenhouse experiment, the treatment with ME-LCO stood out in relation to the other treatments, with a significant increase in NDW, and the same trend was observed in the other parameters evaluated, although with no statistical significance (Table 2). Considering the field experiments, we observed that the SI + ME-LCO treatment contributed to yield gains in both experiments. In the 2018/2019 crop season, the addition of the ME-LCO resulted in yield 7.6% higher than the standard inoculation and was the only treatment statistically equal to the one that was fertilized with 200 kg N $ha^{-1}$. In 2019/2020, although the ME-LCO was statistical equal to the SI, a 3.1% increase in yield was observed. In addition, ME-LCO was the only inoculated treatment that differed from the non-inoculated control (Figure 6).

The heat map and the similarity dendrogram (Figure 4) are in agreement with these results since they showed that supplementation of the standard inoculant with ME-LCO differed from the SI and other treatments supplemented with the molecules, remaining in an isolated cluster associated with greater grain yields. Although the treatment with nitrogen fertilization stood out in both experiments, the use of 200 kg N $ha^{-1}$ is unsustainable, due to its high cost and polluting potential (Moretti et al. 2018).

Similar results were reported in soybean inoculated with *Bradyrhizobium* spp. and *A. brasilense* and supplemented by secondary metabolites of *R. tropici* CIAT 899 and B. *diazoefficiens* USDA 110, that promoted greater yield and increased the number and dry weight of nodules and dry weight of shoots and roots when compared to the treatment with standard inoculation. The authors suggest that secondary metabolites promoted greater root development, which results in increased access to nutrients and water (Moretti et al. 2020a). In another similar study, it was observed that soybean co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense* strains Ab-V5 and Ab-V6 and enriched with metabolites of *B. diazoefficiens* strain USDA 110 and *R. tropici* strain CIAT 899 promoted greater root development, activity, nodulation and an increase in grain yield of 485 kg $ha^{-1}$, 10% higher than the standard inoculation (Moretti et al. 2020b).

Furthermore, supplementation of the standard inoculant with ME-LCO is also associated with protection from abiotic stress conditions (Schwinghamer et al. 2015). The standard inoculation supplemented with ME-LCO extracted from *B. diazoefficiens* USDA 110 and *R. tropici* CIAT 899 positively affected stomatal opening and conductance in soybean, improving the water use efficiency (Moretti et al. 2021). In addition, beneficial effects were observed with the combined use of *Bradyrhizobium* spp., ME-LCO and *A. brasilense* in soybean grown under water stress conditions with an increase in grain yield of 574 kg $ha^{-1}$, about 12.2% higher when compared to inoculation with *Bradyrhizobium* spp. alone (Moretti et al. 2021).

The chemical analysis of the ME-LCO, showed that it is composed of a variety of molecules such as LCOs, COs, EPS, flavonoids, sugars, and growth regulators. According to the results observed in this study, we attribute the gains with ME-LCO to the presence of LCOs and to the other compounds, which we believe have acted together to promote plant growth and increased productivity. This result is consistent with previous studies that showed that in submicromolar doses, the purified LCOs act positively on plant growth and productivity (Khan et al. 2008; Schwinghamer et al. 2016). Although the molecules used in this study have a heterologous origin, the LCOs act in eliciting diverse physiological responses and have hormone-like activity, e.g., changing the root architecture (Prithiviraj et al. 2002; Rosier et al. 2018). In addition, ME-LCO is also composed of apigenin, which is an isoflavonoid that induces the expression of nodulation genes in *B. japonicum* (Brechenmacher et al. 2010).

We observed that supplementation of the standard inoculation with purified CO and EPS showed only occasional positive responses in the field experiments. Although in this study the supplementation of the standard inoculant with the purified molecules did not increase the grain yield of soybeans, we observed isolated effects on NN and NDW using EPS and on TNS using COp. It's known that the EPS is important for nodulation (Staudt et al. 2012; Castellane et al. 2015; Ghosh and Maiti 2016), and that CO induces formation of nodular primordia (this study) and stimulates physiological responses in the host plants (Khan et al, 2011). However, how these molecules act to benefit the growth and production of plants in the field is not fully understood and additional investigations are needed.

**Conclusions**

The standard inoculation of soybean with *Bradyrhizobium* spp. when enriched with total metabolic extract containing nodulation factors (lipochitoligosaccharides) from *Rhizobium tropici* CIAT 899 (ME-LCO) showed the most promising results in greenhouse and field experiments, leading to gains in grain

yield. Possibly, the set of different molecules present in the ME-LCO acted synergistically improving the responses to inoculation.

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# Chapter 3 – Soybean growth promotion by seed and leaf inoculation with secondary metabolites of *Rhizobium tropici* CIAT 899

*"Considérate viejo cuando tengas más recuerdos que sueños"*

*Diego Velázquez (pintor sevillano)*

**Abstract**

Nowadays, there is a growing interest in the use of sustainable technologies for agricultural production. The use of inoculants for growing soybean (*Glycine max* [L.] Merr.) and other crops of interest, as well as the use of secondary metabolites in formulations, is increasing every year. In this study, we investigated the effects of the inoculant containing *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 enriched with secondary metabolism molecules (SMM) containing lipochitoligosaccharides (ME-LCO) and exopolysaccharides (EPS) from *Rhizobium tropici* CIAT 899 in seed and foliar applications in a greenhouse experiment and two field experiments (2019/2020 and 2020/2021). Inoculants containing the molecules were applied via seed as well as with an additional application of the molecules at the V4 stage of development. In the greenhouse, shoot dry weight (SDW) increased by 40.4% in the treatment with EPS foliar application compared to the standard inoculation (SI) with only *Bradyrhizobium* spp. Root dry weight (RDW) increased by 52.5% and 47% in the treatments with EPS foliar application and ME-LCO, respectively, compared to SI, and a significant increase in the number of nodules was observed in the treatment with foliar application with ME-LCO compared to the others. In the field, the treatment that received enrichment with ME-LCO only in the seed stood out from the others in the 2020/2021 crop with an 8.6% increase in productivity compared to the treatment with standard inoculation. The results show that the enrichment of inoculants with molecules from the secondary metabolism of *R. tropici* CIAT 899 is a promising alternative for the development of a new generation of inoculants.

**Key words**: Nod factor, sustainable agriculture, rhizobia, *Bradyrhizobium*

**Introduction**

Biological nitrogen fixation (BNF) corresponds to the reduction of atmospheric $N_2$ to ammonia, a form of nitrogen that can be assimilated by plant metabolism. The symbiosis between legumes and bacteria, called rhizobia, occurs in specialized structures formed in the root of the host, the nodules, where 60% of the nitrogen required by global agriculture is synthesized (Kidaj et al. 2012; Kumar et al. 2019; Liang et al. 2014).

The establishment of symbiotic legume-rhizobia association and root colonization is accompanied by an exchange of molecular signals that lead to a change in host root architecture, which is necessary to ensure nodulation and promote maximum efficiency of BNF (Morel et al. 2016). Flavonoids are the first molecular signal exchanged during symbiosis. They are secreted from the root of the host plant into the soil and act to activate the expression of nodulation genes (nod genes) in the bacteria. The transcription factor nodD, which is constitutively expressed in rhizobia, recognizes flavonoids, and then coordinates the production of nodulation factors (nod factors) or lipochitoligosaccharides (LCOs) (Morel et al. 2016; Oldroyd 2013; Sun et al. 2015). Recognition of LCOs leads to physiological responses in the host such as depolarization of the cell membrane, alkalinization of the intracellular milieu, calcium influx, deformation of root hairs, changes in cytoskeletal organization, and changes in cortical cells to form an active nodule (López-Lara et al. 1995; Morón et al. 2005). In addition to LCOs, other molecules produced by rhizobia also play an important role in symbiotic interactions. Polysaccharides produced by different rhizobia are involved in the nodulation process and may participate in signal transduction necessary for the progress of nodulation and also inhibit plant defense mechanisms (Acosta-Jurado et al. 2021; Staudt et al. 2012). Exopolysaccharides (EPSs) are acidic polysaccharides that, when secreted by rhizobia, have multiple functions, such as protection against environmental stress, aggregation on biotic and abiotic surfaces, and function as a carbon source, in addition to being key molecules for infection of different rhizobia species (Acosta-Jurado et al. 2021)

LCOs are not only key molecules in nodule formation but are also involved in other physiological processes in addition to nodulation (Khan et al. 2008). Because they act mitogenically and directly stimulate plant growth, nodulation factors act as plant growth regulators and stimulate the development of lateral roots and root hairs in both host and non-host plants (Kidaj et al. 2020; Lian et al. 2002; Souleimanov et al. 2002). When applied exogenously, LCOs affect lateral root formation in *Medicago truncatula* (Oláh et al. 2005) and, at submicromolar concentrations, increase germination and nodulation of soybean (Souleimanov et al. 2002). In a work conducted by Marks et al. (2013), a 4.8% increase in soybean yield (*Glycine max* [L.] Merr.) was observed when seeds were inoculated with *Bradyrhizobium* spp. enriched with a bacterial metabolic extract containing LCO, flavonoids, and other secondary metabolic molecules such as EPS. Much of the research that has been done with the use of secondary metabolism molecules has been done with application via seeds. However, there are studies showing that foliar application of LCO increases photosynthetic rate in soybean and maize (Khan et al. 2008) and also causes an increase in shoot and dry weight in maize (Prithiviraj et al. 2003). Poodleśny et al. (2014) observed that LCO extracted from *R. leguminosarum* bv. *viciae* applied through leaves on peas increased chlorophyll concentration, photosynthetic intensity, and grain yield.

Recently, our research group reported that the supplementation of a commercial soybean inoculant containing *Bradyrhizobium* spp. with a total metabolic extract of *Rhizobium tropici* CIAT 899 containing

LCOs, flavonoids, and other molecules increased the grain yield of soybean in two different crops by 7.6% and 3.1% compared to the control inoculated with *Bradyrhizobium* spp. only (Bomfim et al. 2021). In the present study, the application of the total metabolic extract of *R. tropici* CIAT 899 containing LCO (ME-LCO) and EPS in soybean via seed as an adjunct to a commercial inoculant containing elite strains of *Bradyrhizobium* spp was evaluated. It was investigated whether the additional application of the molecules via foliar promotes higher plant growth and productivity. The hypothesis of this work is that the addition of molecules through the leaves can promote the growth of soybean during the growing season and consequently increase grain yield. Therefore, one experiment was conducted in the greenhouse and two experiments were conducted in the field with different crops to determine how to best apply the molecules of *R. tropici* CIAT 899 in soybean.

**Material e Methods**

*Bacterial strains and extraction of secondary metabolites of Rhizobium tropici CIAT 899*

Soybean inoculation was performed with a commercial product containing the strains *Bradyrhizobium japonicum* SEMIA 5079 (= CPAC 15) and *B. diazoefficiens* SEMIA 5080 (= CPAC 7). The strain *R. tropici* CIAT 899, known for the synthesis of a variety of LCOs (del Cerro et al. 2015; Marks et al. 2013, 2015), was used for the extraction of secondary metabolites. The strains used in this work are deposited in the Culture Collection of Multifunctional Microorganisms at Embrapa Cerrados (Brasília, Distrito Federal - Brazil).

The extraction of LCO-containing secondary metabolites (ME-LCO) from *Rhizobium tropici* CIAT 899 was performed according to Marks et al. (2013, 2015). The strain was cultured in minimal B-medium enriched with apigenin (1.0 µl $ml^{-1}$) for 48 hours and maintained under constant agitation at 180 rpm and 28°C. The secondary metabolites were extracted with the organic solvent n-butanol, forming two fractions. The organic phase was kept in a rotary evaporator until completely dry. The resulting material was eluted in 20% acetonitrile to give ME-LCO. EPS was extracted according to Staudt et al (2012) using 1% mannitol as carbon source and ethanol as conducting agent.

*Inoculation methods and treatments*

Three inoculation methods were compared in this study: (1) standard inoculation (SI) in seed with *Bradyrhizobium* spp. only; (2) SI in seed supplemented with ME-LCO and EPS; and (3) SI in seed supplemented with ME-LCO and EPS and additional inoculation of molecules via foliage (FI) at developmental stage V4. The treatments evaluated in the greenhouse and in the field were thus: (i) control without inoculation and without fertilization, (ii) control without inoculation and with nitrogen fertilization corresponding to a dose of 200 kg $ha^{-1}$ N; (iii) SI; (iv) SI + ME-LCO; (v) SI + EPS; (vi) SI + ME-LCO + FI (ME-LCO); (vii) SI + EPS + FI (EPS).

The inoculant was applied to provide $1.2x10^6$ cells $seed^{-1}$ of *Bradyrhizobium* spp. as recommended in Brazil to ensure maximum efficiency of the product. ME-LCO and EPS were diluted in the liquid inoculant to a concentration of 1µl $ml^{-1}$ and 62.5 µl $ml^{-1}$, respectively, when inoculated via seed. For foliar application, 3µl $ml^{-1}$ and 187.5 µl $ml^{-1}$ of ME-LCO and EPS, respectively, were diluted in distilled water.

*Experiment in the greenhouse*

The experiment was conducted in Leonard jars, with a sterilized substrate of sand and charcoal (1:1, v/v). Fertilization was carried out with the modified Norris nutrient solution, which did not contain nitrogen (Norris and Mannetje 1964). The experiment was conducted with genotype RK6316 I PRO Intacta (KWS) commercial seeds. Seeds were previously disinfected according to the Vicent protocol (1970), consisting of thirty seconds in 90% alcohol and five minutes in a 25% sodium hypochlorite solution, with sequential washings with autoclaved distilled water. Then inoculated according to the recommendations of the manufacturer. Sowing was done one hour after inoculation.

Jars were grouped in a randomized block experimental design with seven treatments and four replicates. Three plants were planted in each pot and three days after complete germination (DAG) the seeds were thinned, and the number of plants was standardized to two in each pot. Foliar application was made after the third clover leaf had fully opened during growing season V3 (Fehr and Caviness 1977). The final volume of the solution was 1mL (water and molecule) for each container of two plants at the previously mentioned concentration. The molecules were applied by spraying over the leaves.

Shoot dry weight (SDW), root dry weight (RDW), and nodule number (NN) and dry weight (NDW) were evaluated. The nodules were separated from the roots and dried at a temperature of 40ºC for 72 hours and then counted. Plant roots were washed and dried at 65ºC for at least 72 hours and then weighed. All analyzes were performed on precision balances. Leaves were used for determination of total N by the Berthelot perchloric digestion method (Woolley et al. 1960).

*Experiments in the field*

*Area description*

Two experiments were conducted in the 2019/2020 and 2020/2021 crop seasons. The experiments were conducted in the experimental area of the Cerrado Agricultural Research Center (CPAC), located in Planaltina, Distrito Federal, Brazil (15º 35' 30" S and 47º 42'30" W, altitude of 1,175m above sea level). According to the Köeppen classification, the climate of the region is of the Cwa type, typical of the Savannah. It is characterized by a rainy summer that lasts from October to April and a dry winter that occurs between the months of May and September (Lopes et al. 2013). The soil is described as Acrustox (Oxissol) with clayey texture (513 g $kg^{-1}$ clay, 186 g $kg^{-1}$, silt and 301 g $kg^{-1}$ sand). The climatological data collected during the period of conducting the experiments and soil analysis are presented in Figure 8 and Table 4, respectively.

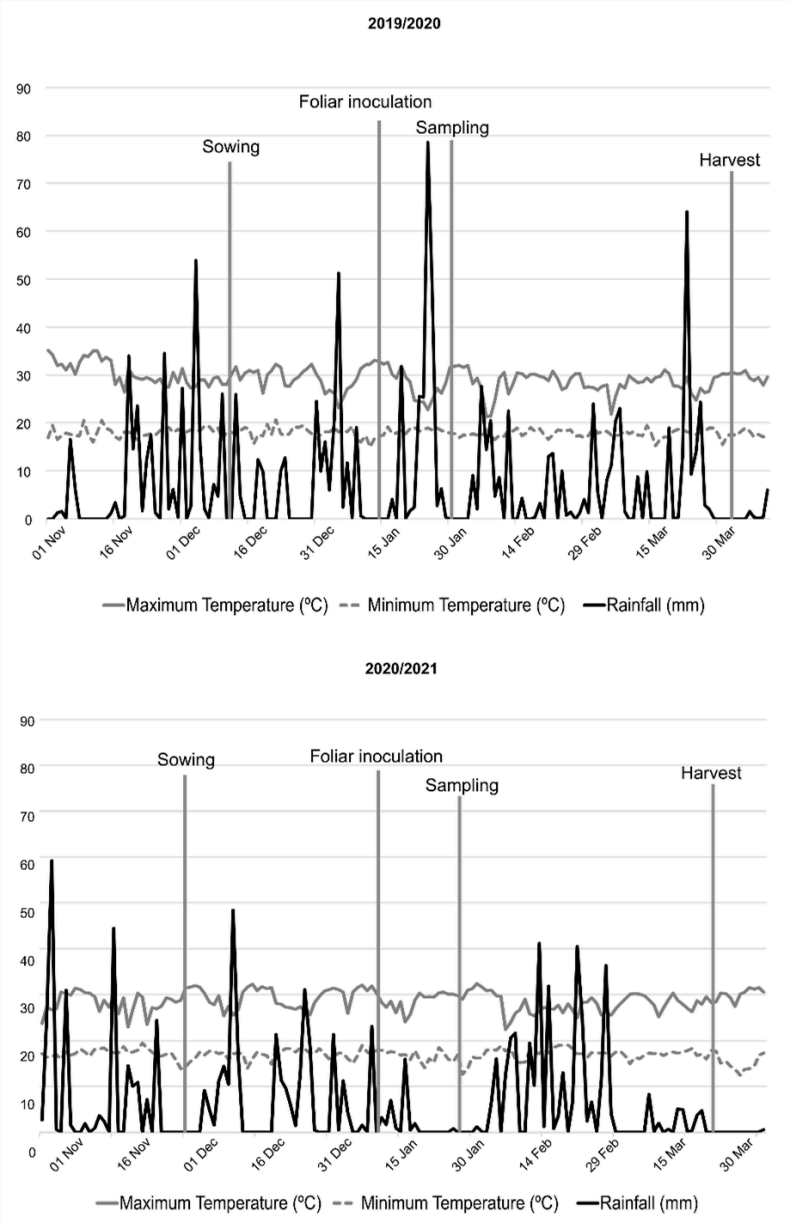


**Figure 8.** Rainfall and maximum and minimum temperatures in the experimental area of Embrapa Cerrados during the period of the experiments conducted 2019/2020 cropping season and 2020/2021 cropping season.

**Table 4.** Chemical characteristics of the soil in the experimental area of the Cerrado Agricultural Research Center (CPAC). Soil samples were taken from the 0-20 cm layer and all analyses were performed before sowing.

| | | Al | Ca | Mg | H+Al | K | P | OM | V |
|---|---|---|---|---|---|---|---|---|---|
| Crop season | pH | ---------- | $cmol_c.dm^{-3}$ | ---------- | | -------- | $ml.L^{-1}$ | ----- | ------- % ------- |
| 2019/2020 | | 0.121 | 1.675 | 0.748 | 5.389 | 144.16 | 18.14 | 2.54 | 34.08 |
| 2020/2021 | | | | | | | | | |

OM; soil organic matter. V; bases saturation = [(K + Ca + Mg)/CEC] × 100. CEC; cation exchange capacity (SB + H + Al).

*Experimental design and execution of the experiment*

The experiments were conducted in randomized blocks of five replicates. Soybean seeds Brasmax Desafio 8473 RR (Bayer) were used in the 2019/2020 crop while cultivar RK6316 I PRO Intacta (KWS) was used in the 2020/2021 crop. Both genotypes are used in the Central-West region of Brazil and are well adapted to the climatic conditions of the region.

The experimental plots were 4 m (length) x 5 m (width) with eight rows spaced 0.5 m apart. 100 seeds were planted per row. The spacing between plots was 1 m to avoid cross-contamination between treatments. The experiments received 300 kg $ha^{-1}$ of fertilization with NPK (0-20-20). In the nitrogen fertilizer treatment, the amount of urea was divided into four applications, the first after planting and later at developmental stage V4, during flowering and pod filling.

Seeds were inoculated one hour before sowing with a commercial liquid inoculant, following the manufacturer's guidelines to ensure a concentration of 1.2 x $10^6$ cells per seed, which is the recommended concentration in Brazil (Hungria et al. 2017). Foliar fertilization was done manually with a spray when the plants reached the V4 stage of development (Fehr and Caviness 1977). The preparation of metabolites for foliar fertilization was done in distilled water and for a final volume of 150 L $ha^{-1}$. The concentration of the molecules was calculated based on the required volume of inoculant for the planted area and based on this calculation, the molecules were diluted to concentrations of 3 µl $ml^{-1}$ and 187.5 µl $ml^{-1}$ of ME-LCO and EPS, respectively.

*Agronomic analysis*

After reaching stage V4 growth stage, six plants were taken from each plot, three from the second row and three from the seventh row. The plants were used to determine the shoot dry mass (SDW) and nodule number (NN) and dry mass (NDW) as described above. Total N in shoots (TNS) was also determined. After reaching physiological maturity, total N in grains (TNG) and grain yield were determined at 13% moisture. Total N in shoots and grains was analyzed using the Berthelot modified perchlorine digestion method (Woolley et al. 1960).

*Statistical analysis*

All analyzes were performed using R software version 3.6.1 (R Core Team 2020). Results were first subjected to a test for normality and homogeneity of the data. After this analysis, the data were subjected to an analysis of variance (ANOVA) and in case of statistical significance, the data were subjected to a Duncan pos hoc test at $p \leq 0.05$. Statistical analyzes were performed using the ExpDes.pt package. Boxplot type graphs were created using the ggplot2 package.

**Results**

*Experiment in the greenhouse*

Shoot dry weight (SDW) increased significantly (40.4%) under SI supplementation with EPS added to seed and then with FI at developmental stage V3 compared to SI (Table 5). Plants receiving SI + ME-LCO and SI + ME-LCO + FI (ME-LCO) increased SDW by 23.1% and 31.1%, respectively, compared to treatment with SI alone, although there was no statistical difference. Among the treatments enriched with

the molecules, SI + EPS showed the worst performance. The non-inoculated control showed the lowest SDW among all the treatments evaluated (Table 5).

Root dry weight (RDW) was statistically higher in the treatments receiving FI than in the treatment receiving SI (Table 3). SI + EPS + FI (EPS) and SI + ME-LCO + FI (ME-LCO) had a 52.5% and 47% increase in RDW compared to SI. Moreover, SI + ME-LCO showed an increase of 35.36% compared to SI, but with no statistical difference. Similar to SDW, among the treatments with molecule addition, SI + EPS showed a lower RDW, which was statistically equal to that of the control treatment (Table 5).

Nodule number (NN) was significantly higher in SI + ME-LCO + FI (ME-LCO) than in all other treatments investigated in this study (Table 5). SI + EPS + FI (EPS) increased NN by 39.13% compared to SI + EPS. However, there was no statistical difference compared to the other treatments. Nodules were not observed in the control treatment without inoculation (Table 5).

**Table 5**. Shoot dry weight (SDW), root dry weight (RDW), nodule number (NN), nodule dry weight (NDW) of soybean cultivar RK6316 I PRO Intacta (KWS) at 38 days after emergence (DAE) in response to the standard inoculation (SI) with *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080, its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides ME-LCO and exopolysaccharides EPS) and foliar inoculation (FI) with ME-LCO and EPS at 15 DAE. Experiment carried out under greenhouse conditions.

| **Treatments** | **SDW (g plant$^{-1}$)** | **RDW (g plant$^{-1}$)** | **NN (n plant$^{-1}$)** | **NDW (g plant$^{-1}$)** |
|---|---|---|---|---|
| Non-inoculated control | 0.568 $^{d}$ | 0.327 $^{c}$ | 0 $^{c}$ | 0 $^{c}$ |
| Standard inoculation (SI) | 1.348 $^{bc}$ | 0.410 $^{bc}$ | 36.66 $^{b}$ | 0.204 $^{ab}$ |
| SI + ME-LCO | 1.660 $^{ab}$ | 0.555 $^{ab}$ | 42.66 $^{b}$ | 0.238 $^{ab}$ |
| SI + ME-LCO + FI | 1.768 $^{ab}$ | 0.603 $^{a}$ | 62.00 $^{a}$ | 0.243 $^{ab}$ |
| SI + EPS | 1.155 $^{c}$ | 0.355 $^{c}$ | 34.00 $^{b}$ | 0.184 $^{b}$ |
| SI + EPS + FI | 1.893 $^{a}$ | 0.625 $^{a}$ | 37.83 $^{b}$ | 0.256 $^{a}$ |
| **CV (%)** | **28.43** | **27.91** | **39.57** | **27.56** |

Means followed by the same letter on the same column are not significantly different according to Duncan's test ($p \leq 0.05$).
CV coefficient of variation.

*Experiments in the field*

No statistical difference was observed between treatments when TNS and SDW were evaluated in the experiments conducted in the 2019/2020 and 2020/2021 crop years (Figure 9). In the experiment conducted in the 2019/2020 crop, SI + EPS was the only treatment that was statistically superior (31.78%) to SI in terms of NN. SI + EPS + FI (EPS) and SI + ME-LCO showed an increase of 31.5% and 20.7%, respectively, compared to SI, but with no statistical difference (Figure 9). In 2020/2021 crop, SI + ME-LCO + FI (ME-LCO), SI + ME-LCO and SI + EPS showed an increase of 20.5%, 14.13%, and 4.9%, respectively, compared to SI, but no statistical difference was observed among treatments (Figure 9). NDW was significantly lower in the treatment that received only nitrogen fertilization compared to the other treatments inoculated with *Bradyrhizobium* spp. in the 2019/2020 crop season. However, in the 2020/2021 season, the treatment with nitrogen fertilization and SI + EPS + FI (EPS) were not statistically different and showed the lowest NDW among the evaluated treatments. In addition, SI + ME-LCO + FI (ME-LCO) and SI + ME-LCO showed an increase in NDW of 17.37% and 5.9%, respectively, compared to SI, but with no statistical difference (Figure 9).

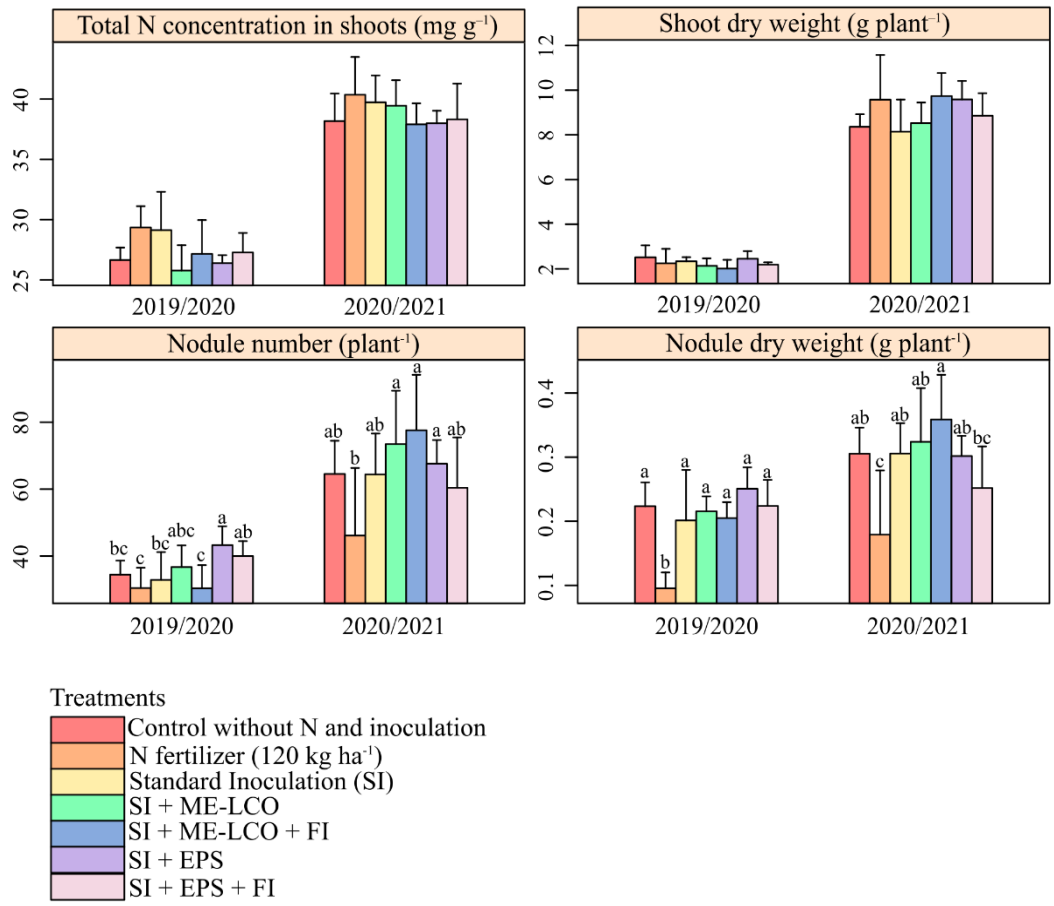


**Figure 9**. Bar chart of total N concentration in shoots (TNS), shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW) at the V4 growth stage of soybean in two experiments (2019/2020 and 2020/2021 crop seasons) located in the experimental field of the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO and exopolysaccharides - EPS) and additional foliar inoculation (FI) with ME-LCO and EPS. Means followed by the same letter and boxes without letters are not significantly different according to Duncan's test ($p \leq 0.05$).

In the 2019/2020 crop, treatments inoculated with *Bradyrhizobium* spp. and supplemented with ME-LCO and EPS molecules and treatments with FI did not differ in grain yield from SI (Figure 10). However, an increase of 167.66 kg ha$^{-1}$ (4.0%) and 153.87 kg ha$^{-1}$ (3.1%) in grain yield was observed in SI + EPS + FI (EPS) and SI + ME-LCO compared to SI, respectively. In 2020/2021, addition of ME-LCO to *Bradyrhizobium* spp. increased grain yield by 370.8 kg ha$^{-1}$ compared to the treatment inoculated with *Bradyrhizobium* spp. only. This result reflects an 8.6% increase in grain yield compared to SI. The other treatments did not differ from SI or the control without inoculation or fertilization. The treatments fertilized with nitrogen fertilizer (200 kg ha$^{-1}$ N) obtained the highest grain yield among all treatments in the two experiments evaluated (Figure 10).

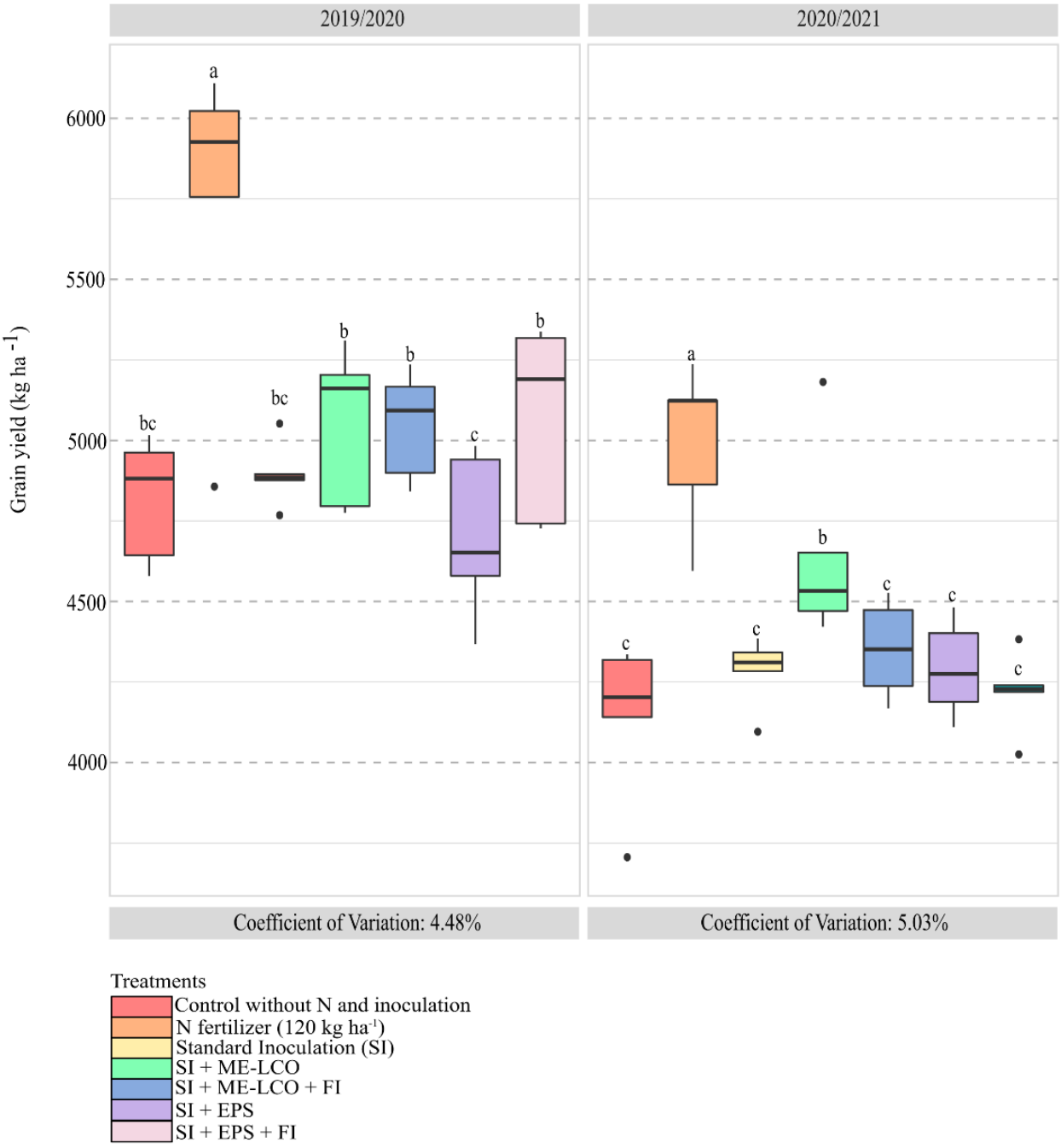


**Figure 10.** Boxplot of grain yield (kg ha$^{-1}$) of soybean in two experiments (2019/2020 and 2020/2021 crop seasons) located in the experimental field of the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO and exopolysaccharides - EPS) and additional foliar inoculation (FI) with ME-LCO and EPS. Lower and upper box boundaries represent 25th and 75th percentiles, respectively; the line inside the box indicates the median; whiskers on the top and bottom represent the 10th and 90th percentiles, respectively; points above and below the whiskers indicate outliers outside the 10th and 90th percentiles, respectively. Means followed by the same letter are not significantly different according to Duncan's test ($p \leq 0.05$)

The similarity dendrogram grouped the grain yield of treatments evaluated in the 2019/2020 and 2020/2021 crop years into three main clusters (Figure 11). A single cluster included the treatments SI + EPS, control without inoculation and without N, SI, SI + EPS + FI (EPS), and SI + ME-LCO + FI (ME-LCO). However, treatments SI + ME-LCO and the one that received nitrogen fertilization were grouped in a second and third cluster, respectively.

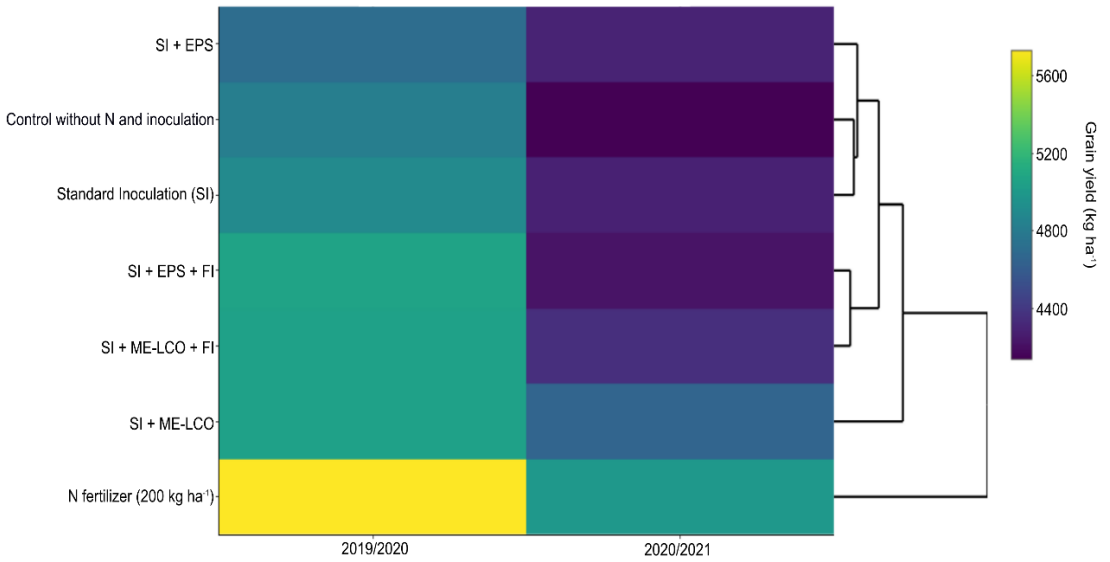


**Figure 11.** Heat map and dendrogram of the Pearson's correlation matrix for the grain yield (kg $ha^{-1}$) of soybean in two experiments (2019/2020 and 2020/2021 crop seasons) located in the Cerrado Agricultural Research Center (Embrapa Cerrados), standard inoculation (SI) with *Bradyrhizobium japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO and exopolysaccharides - EPS) and foliar inoculation (FI) with ME-LCO and EPS.

**Discussion**

Recent data show that in the last 40 years, the total area under soybean cultivation in Brazil has increased from 6.9 million hectares in 1976/1977 to about 36.9 million hectares in the 2019/2020 crops years (Hungria et al. 2020). Currently, Brazil is the largest consumer and producer of soybean inoculants, with about 78% of the cultivated area inoculated annually (Santos et al. 2019). Soybean production in Brazil has increased the demand for bio-based products that ensure high productivity, combined with lower costs and reduced environmental impacts, such as lower greenhouse gas emissions and lower risk of groundwater contamination. The use of molecules derived from the secondary metabolism of rhizobia is a promising strategy to increase the efficacy of inoculants present on the market. However, few studies have been conducted to determine the best method for applying these molecules in the field. The results reported in this study may help to understand the inoculation of molecules and be useful for the development of new formulations.

In the greenhouse experiments, we observed that foliar application with EPS caused a significant increase in SDW and RDW compared to treatment with *Bradyrhizobium* spp. only. Although there was no statistically significant difference, the treatments receiving supplementation with ME-LCO and the treatment with its foliar application stood out compared to SI in the parameters SDW, RDW, and NDW,

while SI + ME-LCO increased NN significantly compared to all the treatments evaluated. When *Bradyrhizobium* spp. inoculation was supplemented with ME-LCO (seed only and also with additional foliar inoculation) and with seed and foliar EPS, in general, all evaluated parameters related to growth promotion were increased (Table 5).

The plant growth promotion by metabolites secreted by microorganisms has been discussed in several scientific papers. For instance, *Azospirillum brasilense* is the most studied plant growth-promoting bacterium and is currently recommended in Brazil for inoculation of grasses such as maize and wheat (Hungria et al. 2010) and co-inoculation with soybean (Hungria et al. 2013). Its ability of plant growth promotion is attributed to the production of growth regulators such as indoleacetic acid (IAA), which acts on lateral root growth and significantly increases the plant's ability to absorb water and nutrients (Fukami et al. 2016). Other molecules of secondary metabolism of rhizobia such as LCO, EPS, flavonoids, IAA present in the metabolic extract are also associated with increased inoculum survival, greater infectivity as well as growth promoting effects on the host plant (Marks et al. 2013).

In this work, we observed that in the two crops studied, ME-LCO increased grain yield compared to the treatment inoculated only with *Bradyrhizobium* spp (Figure 10). Similarly, Marks et al. (2013) observed a 4.8% increase in productivity when soybean was inoculated with *Bradyrhizobium* spp. enriched with secondary metabolites from *B. diazoefficiens* USDA 110. The authors report that the yield increases are associated with the presence of molecules that benefit the plant development, such as flavonoids (from the culture medium enriched with apigenin), EPS, growth regulators and LCO

Flavonoids are the major signaling molecules between rhizobia and the host and regulate the expression of genes associated with the production of LCOs (Janczarek et al. 2014). Rhizobia inoculation in the presence of flavonoids increases nodulation, nitrogen fixation, and helps to promote growth, especially in legumes (Morel et al. 2016). Zhang and Smith (1995) observed that the period between inoculation and root hair curling was shortened and greater expression of *nod* genes occurred when inoculated with *B. japonicum* in the presence of the flavonoid genistein rather than when *B. japonicum* was without the flavonoid. The authors observed that shortening the period required for nodule formation anticipates the period of biological fixation, thus increasing the efficiency of the process. Miransari et al (2013) discuss that inoculation with flavonoid ensures communication between the rhizobia and the host plant, leading to efficient nodulation and successive steps to nitrogen fixation. The use of flavonoids is an interesting strategy for cultivation in soils under abiotic stress conditions (osmotic, saline, pH, or temperature) where communication between host plant and rhizobia may be impaired and flavonoid supplementation can be used as a strategy to mitigate the effects of stress.

The role of EPS in rhizobia-plant interactions is still poorly understood. However, EPS is essential for biofilm formation, and its production is regulated via quorum sensing. It acts in the physical protection of biological agents and in the regulation of genes involved in bacterial motility (Castellane et al. 2014) and is also involved in maintaining host plant symbiosis with rhizobia and protecting bacteria from unfavorable conditions, which increases cell viability after inoculation (Tewari et al. 2020). According to Tewari et al. (2020), the addition of EPS to the inoculant containing *Bradyrhizobium* sp. provides protection to the rhizobia from abiotic and biotic stresses and serves as a carbon source for the cells. Therefore, the addition of EPS to the inoculant can increase root infection and improve nodulation efficiency. However, the positive results of EPS addition to the inoculant were only observed in the treatment that received foliar

inoculation of the molecule. It is possible that the molecule has beneficial physiological effects on soybean in addition to its effect of protecting rhizobia in the soil, although this is still unknown.

LCOs are responsible for various physiological changes in the host plant. In legumes, symbiosis with rhizobia depends on signaling via LCOs to create changes in the root that allow their penetration into the root cortical region and promote the nodulation. It is currently well established in the literature that LCOs have high biological activity in inducing calcium influx into the intracellular environment and deformation of root hairs. In cortical cells, LCOs induce the expression of genes responsible for the production of nodulins, which are necessary for cell division and the formation of the nodule primordium (Lerouge et al. 1990; Morón et al. 2005; Oldroyd 2013). LCOs not only play a key role in the rhizobia-legume symbiosis but also act as growth-promoting molecules in a variety of plants, including non-legumes (Liang et al. 2014; Marks et al. 2015; Prithiviraj et al. 2003). In general, LCOs, when applied exogenously, act by stimulating root growth, resulting in greater uptake of water and nutrients, and promoting nodulation (Schwinghamer et al. 2015; Smith et al. 2015). Prithiviraj et al. (2003) observed that application of LCO Nod Bj V (C18:1, MeFuc) extracted from *B. japonicum* 532C increased germination of various crops such as soybean, maize and common bean in greenhouse and field experiments. In a similar study, Souleimanov et al. (2002), using the same molecule, observed an increase in biomass of soybean and maize with an increase of up to 44% in soybean root surface area. Furthermore, LCOs at submicromolar concentrations increased the number of nodules in *Medicago truncatula* and increased seed germination (Macchiavelli and Brelles-Mariño 2004).

In contrast to the satisfactory results of ME-LCO supplementation, in this study we observed that foliar application of ME-LCO did not differ from standard inoculation. In soybean, Khan et al. (2008) reported an increase in leaf area, shoot dry weight and total dry weight by foliar application of LCO extracted from *B. japonicum*. The authors attributed the growth gains to the stimulation of photosynthetic rate in treatments with LCOs. Although photosynthetic rate was not evaluated in this work, we did not observe any increase in productivity (Figure 10) and shoot dry weight (Figure 9) in treatments with foliar application with ME-LCO. Marks et al. (2015) observed in maize an increase in shoot dry weight and total nitrogen accumulated in the shoot of the plants inoculated with *A. brasilense* enriched with LCO of *R. tropici* CIAT 899 via seed and foliar. In addition to the physiological growth promoting effects, foliar application of LCOs can also result in reduced recognition of pathogen-associated patterns (PAMPs), allowing colonization by beneficial microorganisms by suppressing plant immune responses (Liang et al. 2013).

In this study, we observed that the additional foliar application of ME-LCO inhibited the beneficial effects observed with seed-only application. The additional application of the molecule may have been harmful to the plant due to the high concentration of metabolites, especially LCO. At concentrations between $10^{-9}$ to $10^{-12}$ M, LCOs are biologically active and cause physiological responses in the plant (Kidaj et al. 2012). At very high concentrations, LCO triggers a self-regulatory mechanism. According to Macchiavelli and Brelles-Mariño (2004), high concentrations of LCO trigger self-regulation, while at concentrations below $10^{-9}$ M, the molecule has a positive effect, for example, by increasing the number of nodules. According to the authors, at high concentrations of LCO, the plant hydrolyzes the molecule in the presence of enzymes and the product of the molecule is not recognized by the receptors. This self-regulatory mechanism serves to control the metabolic cost that symbiosis imposes on the plant (Catford et al. 2003).

In summary, the enrichment of conventional soybean inoculant with ME-LCO from *R. tropici* CIAT 899 increased soybean grain yield. However, the additional application of the molecule via foliar did not increase the parameters for promoting growth and yield.

In conclusion, inoculation with *Bradyrhizobium* spp. in soybean in Brazil is a widely accepted agronomic practice that improves agricultural sustainability without loss of productivity. Technologies to improve the product, currently consolidated in the agricultural market, are promising for the production of new formulations. In this study, we identified an alternative formulation for inoculation of soybean focusing on the method of application of ME-LCO through the seed. The addition of this molecule to conventional soybean inoculants may be a strategy for new formulations.

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# Chapter 4 - Inoculation enriched with molecules from the secondary metabolism of *Rhizobium tropici* CIAT 899 for growth promotion in common bean (*Phaseolus vulgaris* L.)

*"Conhecer a natureza, se integrar a ela e se considerar parte dela é a primeira coisa que se deve ser despertada no cientista"*

*Djalma Martinhão Gomes de Sousa*

**Abstract**

The use of rhizobia in agriculture is an alternative to the use of mineral fertilizers. Currently, a new generation of inoculants, that contain molecules from the secondary metabolism of rhizobia, that act in the symbiosis of the host plant and the microorganism, is being formulated. These molecules enhance the action of commercial inoculants, thus increasing the efficacy of these products. Satisfactory results have already been obtained in crops of agricultural interest such as soybean and maize. However, there are no studies yet for common bean and little is known about the effect of isolated molecules such as lipochitoligosaccharides (LCOs), chitoligosaccharides (CO) and purified exopolysaccharides (EPS). Therefore, the aim of this work was to evaluate the effect of the addition of these molecules to the commercial common bean inoculant containing *Rhizobium tropici* CIAT 899 in laboratory, greenhouse, and field experiments. In this study, the biological activity of purified chitoligosaccharides (COp) in nodule formation in common bean was first evaluated at three concentrations of the molecule. Sterilized common bean seeds were maintained in a Fahraeus solution containing 0.2; 2.0 and 20 mL $L^{-1}$ COp for 10 days, and then the formation of nodule primordia was evaluated. A greenhouse experiment and two field experiments were conducted to evaluate the effects of adding molecules to the standard inoculation (SI) with *R. tropici* CIAT 899. The molecules were extracted from *R. tropici* CIAT 899, namely the total metabolic extract containing LCO (ME - LCO) and the other purified molecules LCOp, COp and EPS. As a result, it was found that the formation of nodule primordia in common bean was dose dependent, with the formation of nodule primordia increasing with increasing COp concentration. In the greenhouse, it was observed that inoculation with *R. tropici* CIAT 899 increased shoot dry weight (SDW) and root dry weight (RDW) compared to the control treatment without inoculation. However, no difference was observed between SI and the other treatments supplemented with the molecules. In the field, there was also no difference in the response of the treatments supplemented with the molecules compared to the treatment with *R. tropici* CIAT 899 only in the evaluations carried out in the vegetative period and after physiological maturity. This work was the first to be carried out with the addition of molecules from the secondary metabolism of rhizobia in common bean under greenhouse and field conditions. Because this is pioneering work, the results presented here are preliminary and not indicative of a lack of response. We have observed in the laboratory that COp in common bean has a dose-dependent biological activity. Dose adjustment may be required for the results observed in the laboratory to be seen in the greenhouse and field.

**Key words:** Nod factors, biofertilizer, inoculant, sustainable agriculture

**Introduction**

Plants form beneficial mutualistic associations with microorganisms to increase the efficiency of acquiring nutrients from the environment (Luginbuehl and Oldroyd 2017). The most studied interaction between plants and microorganisms is biological nitrogen fixation (BNF), particularly the association between legumes and bacteria called rhizobia. Rhizobia form specialized structures in the root region of the host plant, called nodules, in which atmospheric nitrogen is converted into ammonia ($NH_3$), which is a molecule that can be assimilated by the plant (del Cerro et al., 2015; Oldroyd, 2013; Sachs; Quides; Wendlandt, 2018). For the symbiosis to be successful, the symbiotic partners must exchange molecular signals, starting with the production of flavonoids by the plant and in response, the bacteria produce the nodulation factors or lipochitoligosaccharides (LCOs) (del Cerro et al., 2019; Dénarié; Cullimore, 1993; Marks et al., 2015).

LCOs are essential for root infection and nodulation. LCOs are molecules formed from a primary structure, chitoligosaccharides (CO), consisting of a chain of three to five N-acetylglucosamine groups (GlcNAc). LCOs have unsaturations and branches associated with CO with a variety of substituent groups such as methyl, fucosyl, acetyl, and sulfates. The unsaturations and branchings in the chain determine the specificity of the molecule (Dénarié and Cullimore 1993; Oldroyd 2013; Oldroyd et al. 2011). LCOs act in the root of the plant by stimulating the formation of the infection strand, which allows rhizobia to enter the root tissue and is essential for nodular organogenesis (Khan et al. 2008; Oldroyd 2013). In addition to LCOs, COs and exopolysaccharides (EPS) are molecules involved in nodulation. CO is found in the cell wall of fungi and plays a role in immune response in plants and in promoting root growth (Khan et al. 2011). EPS is an important molecule involved in biofilm formation and is essential for nodule colonization, attachment to root hairs, and nodule formation (Castellane et al. 2015).

In addition, LCOs are involved in the process of photosynthesis and increase plant growth and yield (del Cerro et al. 2015). A new generation of inoculants containing elite strains used in commercial products and enriched with molecules derived from the secondary metabolism (SMM) of rhizobia have been studied (Bomfim et al. 2021a; Marks et al. 2013, 2015; Moretti et al. 2021). LCOs have high biological activity and at submicromolar concentrations between $10^{-9}$ and $10^{-12}$ M, they trigger necessary physiological changes for nodulation in the host (Oldroyd et al. 2011). Application of exogenous LCO promotes root growth by acting on the formation of secondary roots and root hairs, as well as increasing germination and the number of nodules, and consequently increasing grain yield (Kidaj et al. 2012; Prithiviraj et al. 2003; Souleimanov et al. 2002). Marks et al. (2013) showed that supplementation of a commercial inoculant containing *Bradyrhizobium* spp. with molecules from the secondary metabolism of *B. diazoefficiens* USDA 110 containing LCOs, CO, EPS and other molecules increased soybean yield by 4.8% and supplementation of an inoculant containing *Azospirillum brasilense* with SMM from CIAT 899 increased maize yield by 11.4%. However, there are still gaps in understanding the effect of the molecules involved in nodulation to promote plant growth separately and how the molecules act to promote plant growth in the field.

*Rhizobium tropici* CIAT 899, hereafter referred to as CIAT 899, is a microsymbiont of common bean (*Phaseolus vulgaris* L.) found in acidic soils of South America (del Cerro et al., 2016; Martínez-Romero et al., 1991). CIAT 899 produces a variety of LCOs in the presence of flavonoids, which act as inducing molecules, and in abiotic stress environments such as saline and acidic conditions (del Cerro et al., 2015). Genome analysis of CIAT 899 shows that the bacterium possesses an operon-containing plasmid

with genes responsible for symbiosis with five copies of the nodD gene and three copies of nodA, which may be responsible for the ability to produce a variety of LCOs under different environmental conditions (del Cerro et al., 2019; Pérez-Montaño et al., 2016). These properties make CIAT 899 a promising strain for the production of secondary metabolites such as LCOs, COs, and EPS.

The common bean is a legume that has the ability to associate with a variety of soil rhizobia. However, biological nitrogen fixation is less efficient in these crops compared to other legumes (Filipini et al. 2020; Michiels et al. 1998). Brazil is one of the main consumers and producers of common beans in the world, but cultivation is still highly dependent on the use of nitrogen fertilization (Bomfim et al. 2021b; Filipini et al. 2020). Currently, it is recommended to inoculate common bean with CIAT 899 in Brazil (Hungria et al. 2003), as it is a strain that is highly competitive with native soil rhizobia, genetically stable and tolerant to acidic soils and high temperatures (Maximiano et al. 2020). However, inoculation of common beans in Brazil is still a little-used practice, applied by only 5% of producers, due to the great diversity of crop responses to inoculation (ANPII 2018; BRASIL 2020). Moreover, BNF frequentlyrreedddsdds005AXDXDCCFCFVVFGHGrfERK does not cover all the nitrogen needs of the crop and nitrogen fertilization is still necessary in the common bean crop along with inoculation. New technologies and strategies for growing common bean have been explored to increase the effectiveness of inoculation and the efficiency of BNF (Hungria et al. 2013).

In the present study, molecules from the secondary metabolism of CIAT 899, such as the total LCO-containing metabolic extract (ME - LCO) and the purified molecules LCO, CO and EPS, were added to the commercial inoculant based on CIAT 899. Supplementation of inoculants with molecules that are important for nodulation and have hormone-like effect may enhance the action of rhizobia and improve the efficacy of the inoculant. Therefore, a greenhouse experiment and two field experiments (in different crop season) were conducted to achieve a better understanding of the combined effect of the strain with secondary metabolism molecules.

## Material and Methods

*Bacterial strains and extraction of secondary metabolites of Rhizobium tropici CIAT 899*

CIAT 899 is registered with the Ministry of Agriculture, Livestock and Supply (MAPA) and is currently recommended for common bean inoculation (Hungria et al. 2003). A commercial product containing the bacterium was used for inoculation in the greenhouse and field experiments, and inoculation was carried out according to the manufacturer's instructions. CIAT 899 synthesizes a variety of LCOs, making it a promising bacterium for secondary metabolite extraction (del Cerro et al. 2015b; Marks et al. 2013, 2015) and was used for SMM extraction in this work. CIAT 899 is deposited in the Culture Collection of Multifunctional Organisms at Embrapa Cerrados (Brasília, Distrito Federal - Brazil).

The extraction of LCO from CIAT 899 was performed according to Marks et al. (2013, 2015). The strain was grown for 48h in minimal medium B supplemented with inducing flavonoids. Nodulation factors and CO were extracted with the organic solvent n-butanol, forming two fractions. The organic fraction used to obtain CO and LCO was extracted with solid-phase extraction cartridges and precleared as described by Soría-Díaz et al. (2003). The resulting aqueous fraction containing secondary bacterial metabolites that are not soluble in butanol forms the total metabolic extract (ME-LCO), which was also used in this study. Concentration and lyophilization of the molecules were performed as described by Guasch-Vidal et al.

(2013). The purified LCO and CO, named LCOp and COp, respectively, and ME-LCO were resuspended in a 20% acetonitrile solution.

*Biological activity of COp*

Seeds were sterilized in 98% alcohol for 30 seconds, then soaked in 3% sodium hypochlorite for 5 minutes and washed with sterile water (Vicent 1970). Seeds were pre-germinated in agar-water medium for 72 hours and the seedling was placed in test tubes containing 30 ml Fahraeus solution (Fahraeus 1957) with a metal grid as support. The molecules were added to the Fahraeus solution in different concentrations of 0.2; 2.0; and 20 mL $L^{-1}$ COp or approximately $10^{-9}$, $10^{-8}$, and $10^{-7}$ M COp. Plants were grown for ten days in a growth chamber with 16 hours of light at 26ºC and 8 hours of darkness at 18ºC with a constant humidity of 70%. The presence of nodule primordia (biological activity) was determined by bleaching the roots by immersion in 10% sodium hypochlorite and staining with methylene blue (Truchet et al. 1989). The experiment was carried out with six plants per treatment.

*Greenhouse experiment*

The experiment was conducted in a greenhouse in Leonard jars with sterile substrate of sand and charcoal (1:1, v/v). Fertilization was done with the modified Norris nutrient solution (Norris and Mannetje 1964). Seeds were previously disinfected using the previously described protocol and then inoculated according to the manufacturer's recommendations. Inoculation was carried out one hour before sowing and was done to provide about $1.2 \times 10^6$ cells of CIAT 899 per seed. A liquid inoculant was used and the secondary metabolism molecules of CIAT 899 were diluted to the appropriate concentration.

The jars were arranged in an experimental design of randomized blocks with six treatments and five replicates. The treatments were: (i) control without inoculation; (ii) standard inoculation (SI) containing only CIAT 899; (iii) SI + 1.0 µl $ml^{-1}$ ME-LCO; (iv) SI + 0.5 µl $ml^{-1}$ LCOp; (v) SI + 2.0 µl $ml^{-1}$ COp; and (vi) SI + 62.5 µl $ml^{-1}$ EPS. Three plants of common bean cultivar Pérola were planted in each pot. Three days after complete germination (DAG), thinning of the seeds was performed, unifying two plants per pot. The experiment was completed 30 days after germination (DAG) and the plants were used for analysis. Shoot dry weight (SDW), root dry weight (RDW), and nodule number (NN) and dry weight (NDW) were evaluated. The nodules were separated from the roots and dried at a temperature of 40ºC for 72 hours and then counted. Plant roots were washed and stored at 65ºC for at least 72 hours and then weighed. All analyzes were performed on precision balances. Leaves were used for determination of total N by the Berthelot perchloric digestion method (Woolley et al. 1960).

*Experiments in the field*

*Area description*

Two experiments, conducted in the 2019 crop season (dry season) and in the 2020/2021 crop season (rainy season), were carried out in the experimental area of the Cerrado Agricultural Research Center (CPAC) in Planaltina, Federal District, Brazil (15º 35 '30" S and 47º 42'30" W, altitude 1,175m above sea level). According to the Köeppen classification, the climate of the region is of the Cwa type, typical of the savanna. It is characterized by a rainy summer that lasts from October to April and a dry winter that occurs between the months of May and September (Lopes et al. 2013). The soil is characterized by the Acrustox type with clayey texture (513 g $kg^{-1}$ clay, 186 g $kg^{-1}$ silt, and 301 g $kg^{-1}$ sand). The climatological data collected during the period of execution of the experiments are shown in Figure 12.

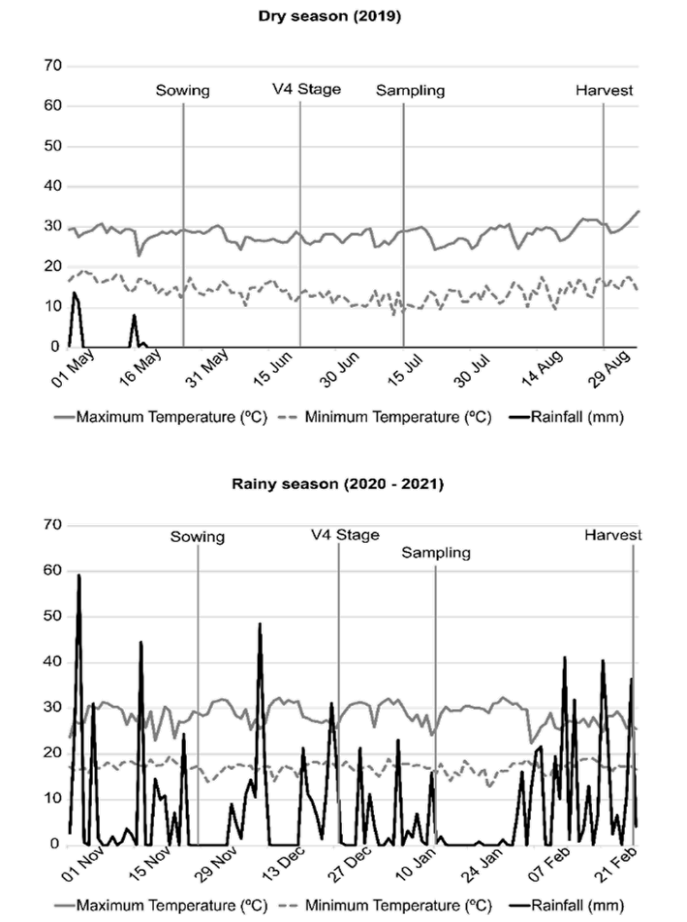


**Figure 12.** Rainfall and maximum and minimum temperatures in the experimental area of Embrapa Cerrados during the period of the experiments conducted in the dry cropping season (2018) and the rainy cropping season 2020/2021.

The number of rhizobia cells capable of nodulating common bean in each experimental plot and the soil chemical characteristics are shown in Table 6.

**Table 6.** Chemical characteristics of the soils in the experimental area of the Cerrados Agricultural Research Center (CPAC). Soil samples were taken from the 0-20 cm layer and all analyses were performed before sowing.

| Crop season | pH | Al | Ca | Mg | H+Al | K | P | OM | V | Common bean rhizobial population[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ---------- $cmol_c.dm^{-3}$ ---------- | | | | -------- $ml.L^{-1}$ -------- | | ------- % ----- | | CFU $g^{-1}$ soil |
| 2019 | | 0.121 | 1.675 | 0.748 | 5.389 | 144.16 | 18.14 | 2.54 | 34.08 | $1.6 \times 10^6$ |
| 2020/2021 | | | | | | | | | | $2.5 \times 10^3$ |

OM; soil organic matter. V; bases saturation = [(K + Ca + Mg)/CEC] × 100. CEC; cation exchange capacity (SB + H + Al).
[1] Most Probable Number Method (MPN) (Vicent 1970)

*Treatments and experimental design*

Experiments were conducted in an experimental design with randomized blocks of six replicates. The experimental plots were 5 m (length) x 2 m (width) with eight rows spaced 0.5 m apart. The spacing between plots was 1 m to avoid cross-contamination between treatments.

The treatments were: (i) control without inoculation with nitrogen fertilization at 50% of the recommended dose for the crop (60 kg ha-1 N); (ii) control with nitrogen fertilization at 100% of the recommended dose for the crop (120 kg $ha^{-1}$ N); (iii) standard inoculation (SI) with *R. tropici* CIAT 899; (iv) SI + 1.0 µl $ml^{-1}$ ME-LCO; (v) SI + 0.5 µl $ml^{-1}$ LCOp; (vi) SI + 2.0 µl $ml^{-1}$ COp; and (vii) SI + 62.5 µl $ml^{-1}$ EPS. All treatments with standard and supplementary inoculation received nitrogen fertilization at 50% of the recommended dose, corresponding to 60 kg $ha^{-1}$ N. Seeds were not previous disinfected. Inoculation with CIAT 899 and preparation of the inoculant containing molecules were carried out as described for the greenhouse test. Nitrogen fertilization with urea was applied after planting, at developmental stage V4, during flowering and pod filling.

*Agronomic analysis*

After reaching V4 growth stage, six plants were collected from each plot, three from the second row and three from the seventh row. Plants were used to determine shoot dry mass (SDW), nodule number (NN) and dry mass (NDW), and the total N in shoots (TNS). These parameters were evaluated as described above. At the end of the production cycle, three plants from the second row and three plants from the penultimate row of the plot were analyzed for number of pods per plant, number of seeds per pod (10 pods) and weight of 100 grains. After reaching physiological maturity, total N in grains (TNG) and grain yield at 13% moisture were evaluated. Grain and leaf N content were determined using the Berthelot modified perchloric digestion method (Woolley et al. 1960).

*Statistical analysis*

All analyzes were performed using R software version 3.6.1 (R Core Team 2020). Results were first subjected to a test for normality and homogeneity of the data. After this analysis, the data were subjected to an analysis of variance (ANOVA) and in case of statistical significance, the data were analyzed using a Duncan pos hoc test at $p \leq 0.05$. Statistical analyzes were performed using the ExpDes.pt package. Boxplot graphs were created using the ggplot2 package.

**Results**

*Biological activity of purified CO*

The increase in COp concentration had an increasing and significant effect on the formation of nodule primordia in common bean. An increase of 66.6% and 75.3% in the number of nodule primordia was observed in the treatment with 20 µl $ml^{-1}$ COp compared to the treatment with 2 µl $ml^{-1}$ and 0.2 µl $ml^{-1}$ COp, respectively (Figure 13).

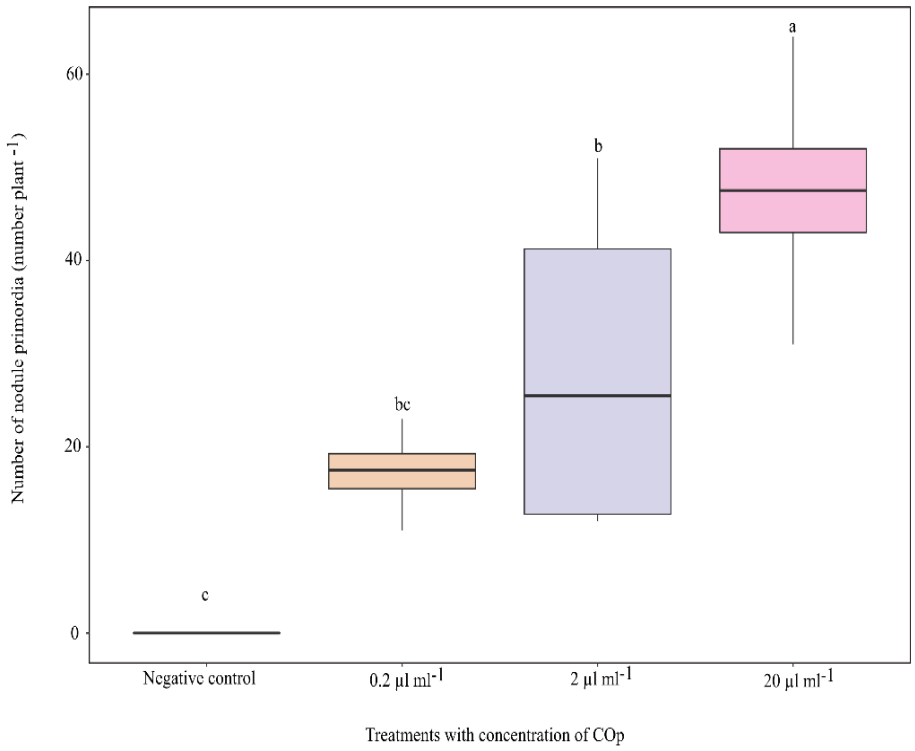


**Figure 13.** Boxplot of the number of nodular primordia in common bean cultivar Perola treated with 0.2 µl $ml^{-1}$, 2.0 µl $ml^{-1}$ and 20 µl $ml^{-1}$ of COp extracted from *Rhizobium tropici* CIAT 899. Lower and upper box boundaries represent 25th and 75th percentiles, respectively; the line inside the box indicates the median; whiskers on the top and bottom represent the 10th and 90th percentiles, respectively; points above and below the whiskers indicate outliers outside the 10th and 90th percentiles, respectively. Means followed by the same letter are not significantly different according to Duncan's test ($p \leq 0.05$).

*Greenhouse experiment*

There was no statistical difference in root dry weight (RDW) between treatments (Table 7). Shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW), and total N in shoots (TNS) showed statistical differences between treatments inoculated with CIAT 899 and supplemented with the secondary metabolism molecules compared to the parameters of the uninoculated control (Table 7). No differences were observed between the treatment with SI and the treatments where the inoculation was supplemented with SMM (Table 7).

**Table 7.** Shoot dry weight (SDW), root dry weight (RDW), nodule number (NN), nodule dry weight (NDW), total N accumulated in the shoot (TNS) of common beans cultivar Perola at 40 days after germination inoculated with *Rhizobium tropici* CIAT 899 (standard inoculation) and its supplementation with molecules of it secondary metabolism (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides - EPS). Experiment conducted under greenhouse conditions.

| **Treatments** | **SDW (g plant$^{-1}$)** | **RDW (g plant$^{-1}$)** | **NN (n plant$^{-1}$)** | **NDW (g plant$^{-1}$)** | **TNS (mg g$^{-1}$)** |
|---|---|---|---|---|---|
| Non inoculated control | 0.318 $^{b}$ | 0.360 | 0 $^{b}$ | 0 $^{b}$ | 10.65 $^{b}$ |
| Standard inoculation (SI) | 1.867 $^{a}$ | 0.423 | 141.3 $^{a}$ | 0.390 $^{a}$ | 36.68 $^{a}$ |
| SI + ME-LCO | 1.713 $^{a}$ | 0.423 | 112.0 $^{a}$ | 0.328 $^{a}$ | 35.10 $^{a}$ |
| SI + LCOp | 1.785 $^{a}$ | 0.420 | 153.8 $^{a}$ | 0.395 $^{a}$ | 35.70 $^{a}$ |
| SI + COp | 2.019 $^{a}$ | 0.475 | 139.7 $^{a}$ | 0.456 $^{a}$ | 34.41 $^{a}$ |
| SI + EPS | 1.712 $^{a}$ | 0.478 $^{ns}$ | 145.4 $^{a}$ | 0.342 $^{a}$ | 34.59 $^{a}$ |
| **CV (%)** | **32.02** | **20.79** | **42.05** | **41.49** | **7.05** |

Means (five replicates) followed by the same letter are not significantly different according to Duncan's test ($p \leq 0.05$)
ns = not significative

*Field experiments*

No statistical differences were observed between treatments when TNS and SDW were evaluated in the trials conducted in the dry and rainy seasons (Figure 14). NN and NDW were lower in the control treatment without inoculation (120 kg ha$^{-1}$ N) compared to the other treatments in the two experiments, but a statistical difference was observed only in the experiment conducted in the dry season (Figure 14).

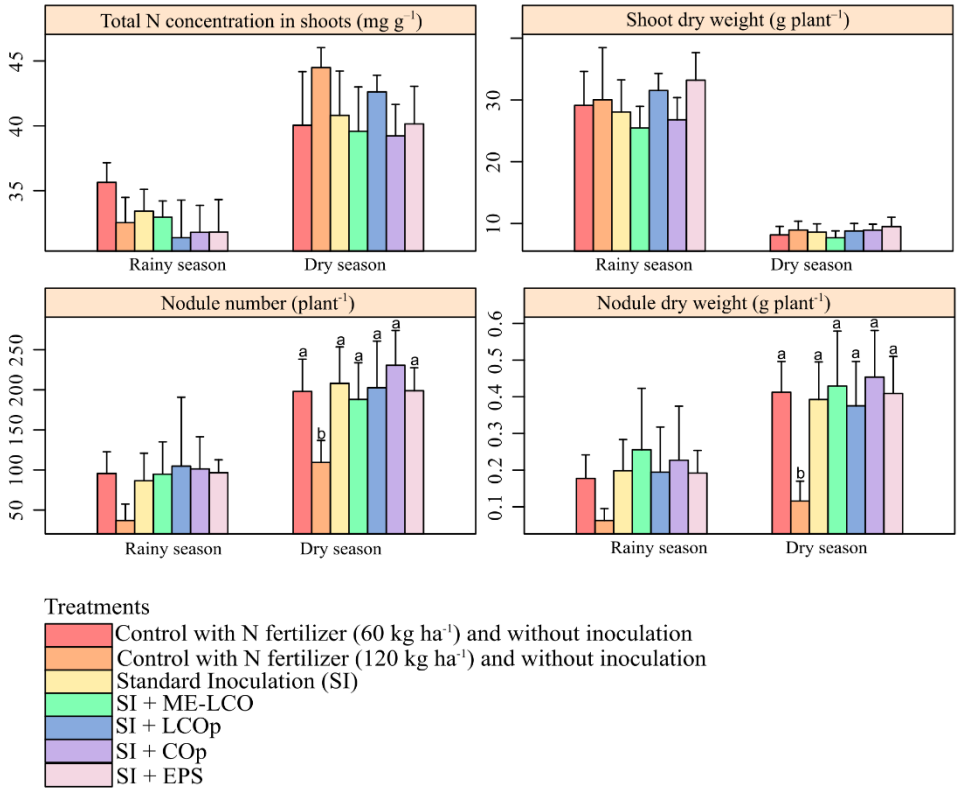


**Figure 14.** Bar chart of total N concentration in shoots (TNS), shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW) at the V4 growth stage of common bean in two experiments (2019 in the dry season and 2020/2021 in the rainy season) located in the experimental field of the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Rhizobium tropici* CIAT 899 and its supplementation with molecules of the metabolism of *R. tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO and exopolysaccharides - EPS) and foliar inoculation (FI) with ME-LCO and EPS. Means followed by the same letter and boxes without letters are not significantly different according to Duncan's test ($p \leq 0.05$).

In the dry season, among the yield components evaluated, a statistical difference was observed only in the number of pods per plant (Figure 15). Although treatments SI + LCOp, SI + COp, and SI + EPS showed a higher number of pods per plant than SI + EPS, it differed from SI with an increase of 30.2%. In the rainy season, the number of grains per pod was higher in SI than the others, but it was not statistically different from SI + LCOp and SI + COp (Figure 15).

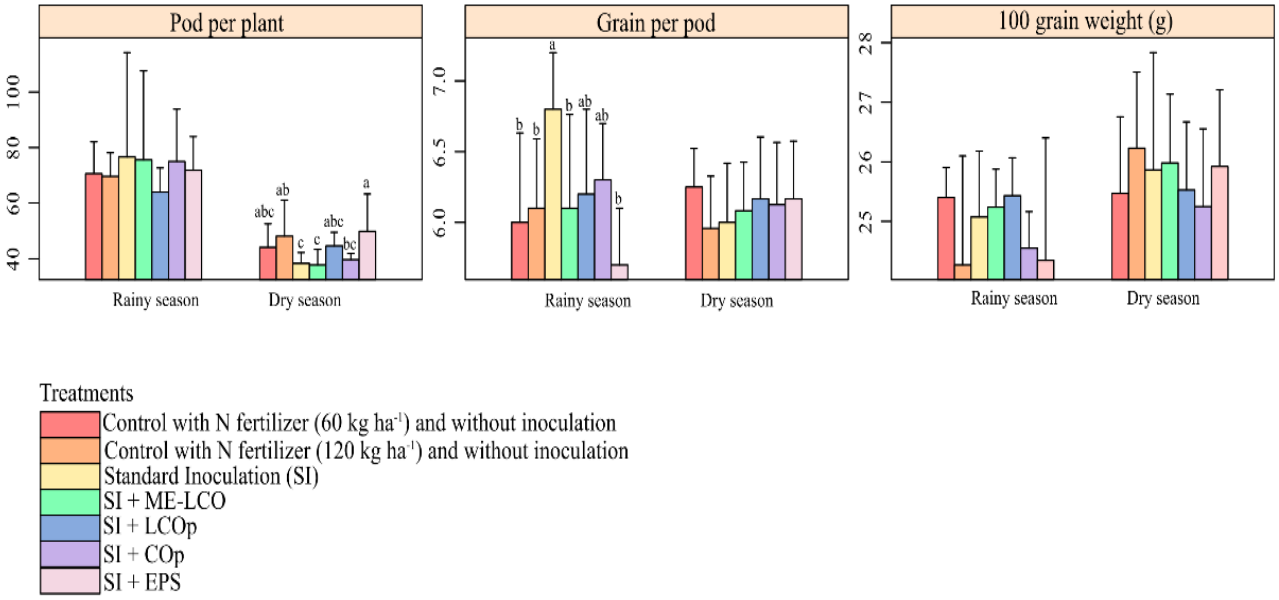


**Figure 15**. Bar chart of yield components being rated pod per plant, grains per pod and 100 grains weight at physiological maturity of common bean in two experiments (2019 in the dry season and 2020/2021 in the rainy season) located in the experimental field of the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Rhizobium tropici* CIAT 899 and its supplementation with molecules of the metabolism of *R. tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO and exopolysaccharides - EPS) and foliar inoculation (FI) with ME-LCO and EPS. Means followed by the same letter and boxes without letters are not significantly different according to Duncan's test ($p \leq 0.05$).

The average grain yield of the experiment conducted in the rainy season did not show statistical differences among treatments (Figure 16). In the dry season, the average grain yield of the control treatment (120 kg ha$^{-1}$ N) stood out from the others with 4326.6 kg ha$^{-1}$, with a difference of 585.9 kg ha$^{-1}$ from SI (Figure 16).

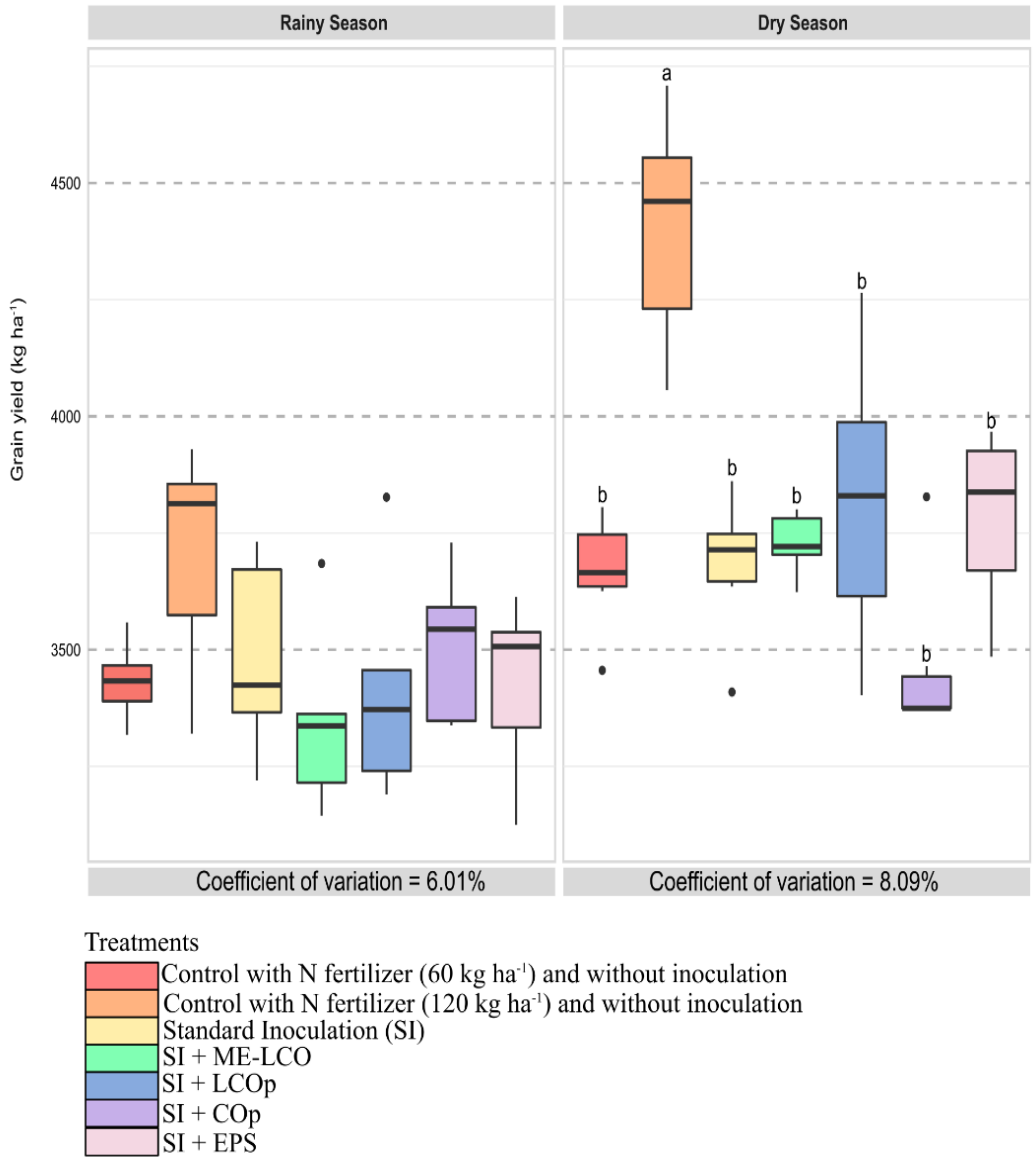


**Figure 16.** Boxplot of grain yield (kg ha$^{-1}$) of common bean cultivar Perola in two experiments (2019 dry season and 2020/2021 rainy seasons) located in the experimental field of the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Rhizobium tropici* CIAT 899 and its supplementation with molecules of the metabolism of *R. tropici CIAT* 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides - EPS). Lower and upper box boundaries represent 25th and 75th percentiles, respectively; the line inside the box indicates the median; whiskers on the top and bottom represent the 10th and 90th percentiles, respectively; points above and below the whiskers indicate outliers outside the 10th and 90th percentiles, respectively. Means followed by the same letter are not significantly different according to Duncan's test ($p \leq 0.05$).

**Discussion**

Nodulation depends on an extensive molecular dialogue between the host plant and its symbiotic partner, and this communication is well described in the literature (Dénarié and Cullimore 1993; Oldroyd 2013). Nodulation factors play a key role in this communication and are crucial for nodule formation in legumes, as they are required for maximal biological nitrogen fixation (Kidaj et al. 2020). These molecules have a mitogenic effect on cortical cells through the induction of genes involved in the plant cell cycle (Souleimanov et al. 2002). Moreover, exogenous application of nodulation factors can increase the number of nodules (Macchiavelli and Brelles-Mariño 2004) and increase the number of lateral roots and root hairs, thereby increasing the root area (Herrbach et al. 2017), suggesting that these molecular signals have a growth-promoting effect in addition to their role in nodulation (Souleimanov et al. 2002). Thus, a new generation of inoculants containing elite strains enriched with molecules has emerged with the aim of improving the quality and efficacy of inoculants already available. Previous studies with formulations containing elite bacteria enriched with LCO and other molecules of rhizobia metabolism has shown promising results in agricultural crops such as soybean (Bomfim et al. 2021a; Marks et al. 2013; Moretti et al. 2020) and maize (Marks et al. 2015), but knowledge of other crops, such as common beans, is still limited.

Although the primary structure of LCOs, COs, has been reported in the literature to be widely distributed in organisms and abundant in shells of crustaceans, insect cuticle, and fungal cell walls (Liaqat and Eltem 2018), the role of this molecule in nodule formation is still uncertain. In the present study, we observed the formation of nodular primordia in the common bean root in a dose-dependent manner of COp, supporting the hypothesis that this molecule has biological activity in common beans. Furthermore, Genre et al. (2013) observed that short-chain CO (C4 – C5) triggers $Ca^{+2}$-mediated signaling and increased intracellular $Ca^{+2}$ influx into *Medicago truncatula* root cells, which is required for nodule organogenesis. COs are involved in the early stages of nodulation (Liang et al. 2014), and in recent studies, Winkler et al. (2020) observed the overexpression of genes related to growth promotion and genes related to nitrogen metabolism in CO -treated *Arabidopsis thaliana*. In previous studies, it was observed that the addition of CO tetraose at concentrations greater than $10^{-8}$ M stimulated the formation of lateral roots and increased mycorrhizal infection in *M. truncatula* (Oláh et al. 2005). In the present work, the biological activity of CO was observed in the formation of nodule primordia in common bean, which is an important indication that this molecule may also be involved in nodule formation.

Under controlled conditions in a greenhouse, it was observed that inoculation with CIAT 899 increased the evaluated parameters for growth promotion compared to the non-inoculated control, but the treatments supplemented with the molecules did not differ from SI in terms of nodulation and growth promotion. These results indicate that the addition of molecules to inoculation with CIAT 899 did not differentiate the response of common bean from SI. This is in contrast to the results of Bomfim et al. (2021a) where it was observed that the addition of ME-LCO of CIAT 899 to the inoculant composed of *Bradyrhizobium* spp. increased NN, NDW, SDW and RDW relative to SI in soybean. The authors found that CIAT 899 molecules, although not a soybean microsymbiont, and containing heterologous molecules, increased all growth promotion parameters evaluated.

The lack of response of the common bean to the inoculation enriched with the molecules is possibly related to the dose of molecules added. It is likely that the concentration of molecules added to the inoculant was insufficient to produce a response in the plant. In a work carried out with the common bean plant, Jesus et al. (2018) observed that co-inoculation of CIAT 899 with *B. diazoefficiens* USDA 110 and *B. elkanii* 29w increased nodulation and biomass accumulation, the increase in these parameters being greater when the cell concentration of *Bradyrhizobium* spp. was higher ($10^8$ CFU $seed^{-1}$), the response of the plant to inoculation being dose-dependent. The authors suggested that co-inoculation with *Bradyrhizobium* spp. was beneficial to common bean as it produced nodulation factors and EPS, which may have helped to increase the number of nodulation sites in the host. Similarly, Carvalho et al. (2020) observed that inoculation of common bean with *Bradyrhizobium* spp. induced nodule formation but did not differentiate into bacteroids. In contrast, co-inoculation of *Bradyrhizobium* spp. with CIAT 899 increased the number of active nodules in the plant and promoted early nodule formation. The authors show that although *Bradyrhizobium* spp. does not form active nodules in the common bean plant, the LCOs produced by the bacteria promoted the formation of active nodules with CIAT 899 in the plant.

It is possible that the perception of secondary metabolism molecules varies depending on the host plant. CIAT 899 produces a wide range of nodulation factors consisting of substituent groups of N-methyl compounds with or without sulfates in the N-acetylglucosamine residues (del Cerro et al. 2019a), and it has been previously reported in the literature that the production of a variety of LCO molecules allows rhizobia to colonize more hosts (López-Lara et al. 1995). Although CIAT 899 is a microsymbiont of the common bean plant and the strain's nodulation factors have biological activity in the plant (del Cerro et al. 2015a), the dose applied in this study may not have been sufficient to produce stimulation in the plant. In experiments with soybean using molecules extracted from CIAT 899 at the same concentrations as in the present work, Bomfim et al. (2021a) observed the response of the legume to inoculant supplementation with ME-LCO in greenhouse and field experiments.

On the other hand, the response in the field differed from that observed in the greenhouse. The treatments with CIAT 899 did not differ from the non-inoculated treatment (60 kg $ha^{-1}$ N) in any of the experiments evaluated, possibly related to the high concentration of native rhizobia in the soil, since the inoculated microorganisms have to compete with the native rhizobia in the soil for colonization of the rhizosphere (Hungary and Mendes 2015; Jesus et al. 2018). According to Vargas et al. (2000), inoculation efficiency in common bean is higher in soils with a low concentration of native rhizobia, as a stronger response to inoculation was observed with the selected strains. However, in soils that have been planted previously and where a native population has become established, inoculation efficiency is lower and nodule occupancy by native rhizobia can reach 90% depending on their concentration in the soil. In addition, native rhizobia can be inefficient in biological nitrogen fixation, which affects nutrient uptake by the plant (Cardoso et al. 2012).

In addition to the native rhizobia community, soil nitrogen concentration has a direct effect on the nodulation efficiency of common bean. It has been clarified in the literature that in soils with high nitrogen concentration, nodulation is inhibited by host self-regulatory mechanisms, reducing BNF (Barros et al. 2016). In this study, it was observed that in the dry season, NN and NDW were significantly reduced in the control treatment without inoculation (120 kg $ha^{-1}$ N) compared to the other treatments. The control without inoculation (60 kg $ha^{-1}$ N) was not different from the other inoculated treatments. These results show that

high doses of nitrogen inhibit the development of nodules in common bean, confirming the work of Vargas et al. (2000). According to Vargas et al. (2000), nitrogen fertilizer doses above 40-60 kg $ha^{-1}$ N inhibit the symbiosis of common bean with the microsymbiont, drastically reducing the efficiency of BNF. According to the authors, the response to common bean inoculation and the need for nitrogen fertilizer varies according to the cropping history of the area.

In conclusion, the results of this study suggest that the SMM of CIAT 899, especially COp, have biological activity in common bean and act in the early stages of symbiosis by affecting the formation of nodular primordia in a dose-dependent manner. As this is pioneering work in common bean, there are still many gaps in our understanding. We believe that the doses used in the greenhouse and field experiments may not have been sufficient to elicit a physiological response in the host. We add that the results presented here are still preliminary and do not indicate a lack of response. The use of different doses may shed more light on the response of common bean to inoculation with secondary metabolites.

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# Chapter 5 – *Azospirillum brasilense* Abv5 and Abv6 supplemented with LCOs extracted from *Rhizobium tropici* CIAT 899 is a promising strategy for formulating a new generation of grass inoculants[1]

*"Pouco conhecimento faz com que as pessoas se sintam orgulhosas. Muito conhecimento, que se sintam humildes. É assim que as espigas sem grãos erguem desdenhosamente a cabeça para o céu, enquanto as cheias as baixam para a terra, sua mãe."*

*Leonardo da Vinci*


**Abstract**

*Azospirillum* is the most studied plant growth promoting bacterium (PGPR) and is currently recommended for inoculation of several crops of agricultural interest such as maize and wheat. The interest in bio-based products has increased significantly in recent years and the use of PGPR has consolidated in the agricultural market. A new generation of inoculants containing commercial bacteria such as *Azospirillum brasilense* enriched with bacterial molecules such as nodulation factors or lipochitoligosaccharides (LCOs), chitoligosaccharides (CO) and exopolysaccharides (EPS) has emerged as a promising strategy. The aim of this study was to evaluate the effects of enrichment of standard inoculation (SI) with *A. brasilense* Abv5 and Abv6 in wheat and maize with secondary metabolism molecules of *Rhizobium tropici* CIAT 899. The molecules evaluated were the total metabolic extract containing LCO (ME - LCO) and the other purified molecules LCOp, COp and EPS. The study was conducted in greenhouse and field experiments. In the greenhouse, wheat cultivars BRS 264 and BRS 394 and maize DKB 290 were studied. Four experiments were carried out in the field, three with maize and one with wheat. In the greenhouse, it was observed that maize treated with standard inoculation (SI) containing *A. brasilense* supplemented with LCOp increased the root dry weight (RDW) by 72.2% compared to the SI treatment. An increase of 59.9% in shoot dry weight (SDW), 17.4% in stem circumference, and 24.8% in height was observed in plants treated with SI + LCOp compared to those treated with SI. The BRS 264 wheat cultivar responded satisfactorily to the SI + LCOp treatment with an increase of 22.4% and 62.5% in SDW compared to the SI treatment and non-inoculated control, respectively. In the field, there was a 10% increase in wheat grain yield with the SI + LCOp treatment compared to the treatment with SI alone in the experiment conducted in the field. In maize, there was no significant increase in productivity of treatments supplemented with the molecules compared to treatment with *A. brasilense* alone. The results observed in this study indicate that the LCOp extracted from *R. tropici* CIAT 899 is promising for supplementing the inoculant based on *A. brasilense*, especially for wheat, these being the first trials conducted in a greenhouse and the field with an inoculant supplemented with bacterial molecules for the culture.



**Keywords:** Nod factors, *Zea mays*, *Triticum aestivum*, secondary metabolites.


[1] A modified version of this chapter will be submitted in the journal Environmental Sustainability in the Special Issue (July-Sept 2022) – Plant growth-promoting microorganism in the New Era: from Ecology to Biotechnology in America

**Introduction**

Nodulation factors or lipochitoligosaccharides (LCO) are signaling molecules produced by rhizobia that play a crucial role in nodule formation during symbiosis with legumes (Poinsot et al. 2016). These molecular signals are part of an important dialog between symbiotic partners that begins with the exudation of flavonoids or isoflavonoids by legumes (Burian and Bensmihen 2018; del Cerro et al. 2019).

LCOs are molecules composed of a chitoligosaccharides (CO) residue formed by four to five N-acetylglucosamine residues with β 1-4 bonds, where the different LCO molecules are reductively composed of methyl, fucosyl, acetyl, and sulfate substituent groups in the subunits (Dénarié and Cullimore 1993; Prithiviraj et al. 2003; Burian and Bensmihen 2018;). The presence of substituent groups at the non-reducing ends formed by fatty acid chains varies in size and degree of saturation, and these factors are important for the specificity of rhizobia with the host plant (Liang et al. 2014; Oldroyd 2013). Molecular arrangements vary depending on bacterial species and environmental conditions, and a single bacterial strain can produce a wide variety of LCO-like molecules (Kidaj et al. 2012).

Nodulation factors act on physiological processes in the host plant that are crucial for nodule organogenesis, such as deformation and bending of root hairs, stimulation of cell division, and induction of nodulins, genes responsible for the production of nodule-specific proteins (Burian and Bensmihen 2018; Minami et al. 1996; Wang et al. 2018). In non-host plants, LCOs are involved in similar physiological responses, but without nodulation, and act similarly to growth regulators (Lian et al. 2002; Prithiviraj et al. 2003). In previous studies, LCOs were observed to stimulate germination of non-legumes (Prithiviraj et al. 2003; Schwinghamer et al. 2015a) and increase leaf area and photosynthetic rate of maize (Khan et al. 2008). According to Kidaj et al. (2012), genes inducible by LCOs are possibly present in legumes and non-legumes, so nodulation factors stimulate growth in non-leguminous plants. Exopolysaccharides (EPS) are involved in nutrient uptake, protection from environmental stress, and adhesion to surfaces between various plant growth promoting bacteria (PGPR) and the host plant root (Castellane et al. 2014). Bacteria of the genus *Rhizobium* produce high concentrations of EPS, as this molecule is essential for biofilm formation and necessary for survival within nodules (Ghosh and Maiti 2016).

*Azospirillum sp.* is currently the most studied PGPR in Brazil and other countries, especially because of its ability to fix nitrogen associatively and produce various growth regulators such as auxins, cytokinins, and gibberellins (Cassán et al. 2020; Tien et al. 1979). In addition, studies on inoculation with the species *A. brasilense* showed greater tolerance of maize to stress conditions (Fukami et al. 2018), greater nutrient accumulation in wheat (Galindo et al. 2019b), and higher productivity in maize (Fukami et al. 2016) and wheat (Galindo et al. 2017). Okon and Labandera-Gonzalez (1994) summarized the results of several field trials conducted over a 20-year period and showed that the increase in productivity of grasses inoculated with *A. brasilense* ranged from 30-70%. Furthermore, inoculation with *A. brasilense* has shown that, in addition to the benefits of promoting plant growth, the producer can reduce total nitrogen fertilizer use in wheat and maize by up to 25% without altering productivity (Fukami et al. 2016; Hungary et al. 2010). In Brazil, the commercialization of inoculants containing *A. brasilense* Abv5 and Abv6 for wheat (*Triticum aestivum*) and maize (*Zea mays*) (Hungria et al. 2010) and *Brachiaria* spp. (=*Urochloa* spp.) (Hungria et al. 2016) started in 2009 (Santos et al. 2019) and, only in 2018, approximately 9 million doses of this biological product were sold (Santos et al. 2019).

New inoculant formulation technologies have been investigated to increase the efficacy of the product. Supplementation of commercial inoculants with molecules of secondary metabolism (SMM), such as LCOs, has been investigated, but although promising, its use is in its incipient. Supplementing *A. brasilense* Abv5 and Abv6 inoculants with an metabolic extract containing (ME-LCO) increased maize yield by 11.4% compared to the standard inoculant (Marks et al. 2013). In another study, it was observed that application of inoculants enriched with ME-LCO of *Rhizobium tropici* CIAT 899 together with *A. brasilense* either via seed or foliar application increased maize yield in five of the six experiments conducted compared to a non-inoculated treatment (Marks et al. 2015). In soybean, a 7.6% increase in grain yield was observed when treated with *Bradyrhizobium* spp. supplemented with the metabolic extract of *R. tropici* CIAT 899 compared to treatment with the standard inoculum alone (Bomfim et al. 2021). However, there are still gaps in the understanding of the effect of purified molecules and there is a need for more results on the supplementation of inoculants with molecules from the secondary metabolism of rhizobia, especially in field trials with different agricultural crops.

In this study, the supplementation of the inoculant based on *A. brasilense* Abv5 and Abv6 with the molecules of secondary metabolism of *R. tropici* CIAT 899 was evaluated, namely the total metabolic extract containing LCO (ME - LCO) and the molecules LCO, CO and purified EPS. The hypothesis of this work is that the addition of these molecules to a commercial inoculant will allow to formulate of more efficient microbial inoculants guaranteeing higher agricultural yield for maize and wheat.

## Material and Methods

### *Bacterial Strains*

Bacterial strains of *A. brasilense* Abv5 and Abv6, registered with the Ministry of Agriculture, Livestock and Food Supply (MAPA) and currently recommended for inoculation of wheat and maize (Hungria et al. 2010), were used for inoculation in the experiments conducted in the greenhouse and in the field. A commercial product containing the bacteria was used and inoculation was carried out according to the manufacturer's instructions. *Rhizobium tropici* CIAT 899 is currently recommended for common bean inoculation in Brazil (Hungria et al. 2003) and synthesizes a variety of LCOs, making it a promising bacterium for SMM extraction. This strain was used in this work for the extraction of secondary metabolites and is deposited in the Culture Collection of Multifunctional Organisms at Embrapa Cerrados (Brasília, Federal District - Brazil).

### *Production and extraction of secondary metabolites from Rhizobium tropici CIAT 899*

The extraction of SMM of *R. tropici* CIAT 899 was carried out according to Marks et al. (2013, 2015). The strain was grown in minimal B medium - supplemented with apigenin at a concentration of 1.0 µl $ml^{-1}$ for 48 hours at a constant temperature of 28ºC and stirring at 180 rpm. Then, the organic solvent n-butanol (1:3, v/v) was used, which promoted the formation of two fractions. The organic fraction was kept in a rotary evaporator and the crude extract was eluted in a 20% acetonitrile solution to obtain the total metabolic extract (ME-LCO). ME-LCO consists of a variety of molecules such as apigenin, sugars, growth regulators and EPS in addition to LCO. Purified lipochitoligosaccharides (LCOp) and chitoligosaccharides (COp) were obtained by purifying the aqueous fraction in reversed-phase thin-layer chromatography (RP-

TLC) using an SPE C18 cartridge according to the protocol described by Guasch-Vidal et al. (2013) and resuspended in a 20% acetonitrile solution. EPS was extracted according to the Staudt methodology (Staudt et al. 2012) using mannitol (1%) as carbon source and ethanol was used for EPS precipitation.

*Experiments in the greenhouse*

Two experiments were conducted in a greenhouse using wheat and maize to evaluate the performance of these crops when inoculated with molecules of secondary metabolism of *R. tropici* CIAT 899 under controlled conditions. Wheat cultivars BRS 264 (Albrecht et al. 2006) and BRS 394 (Albrecht et al. 2015), indicated for the central region of Brazil, were used. For maize cultivation, the commercial variety Dekalb 290 VT Pro 3 (Bayer) was used.

The experiment was conducted in Leonard jars with a sterile substrate of sand and perlite (1:2, v/v). Nutrients were supplied using a modified Norris nutrient solution (Norris and Mannetje 1964) supplemented with the equivalent of 75% of the recommended nitrogen dose for each crop, equivalent to 90 kg $ha^{-1}$ N. Jars were grouped in an experimental design with randomized blocks of five treatments and five replicates, which were a combination of standard inoculation with *A. brasilense* Abv5 and Abv6 supplemented with the SMM (ME-LCO, LCOp and COp). The treatments were (i) control without inoculation and nitrogen fertilization with a dose of 100% nitrogen for the crops (equivalent to 120 kg $ha^{-1}$ N in both crops); (ii) standard inoculation (SI) with *Azospirillum brasilense* Abv5 and Abv6; (iii) SI + ME-LCO; (iv) SI + LCOp; (v) SI + COp and (vi) SI + EPS. The SI + EPS treatment was carried out only with maize in the greenhouse. SI and SI supplemented with molecules received 90 kg $ha^{-1}$ N and 60 kg $ha^{-1}$ N in the experiments with maize and wheat, respectively. In both wheat and maize, inoculation with *Azospirillum brasilense* was done one hour before sowing as recommended by the manufacturer.

Before sowing, the seeds were sterilized according to the Vicent protocol (1970) and allowed to rest for one hour. Then the seeds were inoculated according to the manufacturer's recommendations. The SMM of *R. tropici* CIAT 899 were added to the inoculant at concentrations of 1 ml $L^{-1}$; 0.5 ml $L^{-1}$ and 2 ml $L^{-1}$ of ME-LCO, LCOp and COp, respectively. At the time of sowing, four wheat and three maize seeds were planted and 3 days after germination (DAG) the number of plants was standardized to two in each pot. After 35 and 41 DAG, wheat and maize were harvested, respectively, and then the shoot dry weight (SDW) and the root dry weight (RDW) were evaluated. For maize, stem circumference and shoot height were also evaluated.

*Experiments in the field*

*Description of the area*

Field experiments were conducted at the Cerrado Agricultural Research Center (CPAC) experimental site in Planaltina, Distrito Federal, Brazil (15º 35' 30" S and 47º 42'30" W, elevation 1,007 m). A total of four experiments were conducted, three with maize (two in the 2018/2019 crop and one in the 2019/2020 crop) and one experiment in 2019 (dry season) with wheat (Table 8).

**Table 8**. Agronomic information of experiments conducted in the field area of Embrapa Cerrados (Planaltina, Federal District, Brazil)

| Cultivar | Experiment | Year | Season | Plant genotype | Irrigation[1] | Sowing | Harvest | Line spacing ($m^{-1}$) | Seeds per plot ($m^{-1}$) | Furrows per plot | Planted area ($m^2$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Maize | CPAC 1 | 2018/2019 | Summer | Dekalb 290 VT Pro 3 (Bayer) | No | 21/11/2018 | 16/04/2019 | 0.5 | 30 | 8 | 10 |
| Maize | CPAC 2 | 2018/2019 | Summer | Dekalb 290 VT Pro 3 (Bayer) | Yes | 21/11/2018 | 12/04/2019 | 0.5 | 30 | 8 | 10 |
| Maize | CPAC 3 | 2019/2020 | Summer | 30F53VYH (Pioneer) | No | 04/12/2019 | 26/05/2020 | 0.5 | 30 | 8 | 10 |
| Wheat | CPAC 4 | 2019 | Winter | BRS 264 (Embrapa) | Yes | 13/06/2019 | 19/09/2019 | 0.17 | 90 | 5 | 5 |

[1] The irrigation system was carried out using sprinkler systems

According to the Köeppen classification, the climate of the region is Cwa, characterized by being rainy in summer and dry in winter (Alvares et al. 2013). It has two well-defined periods with a rainy season (with 90% of the expected rainfall for the year) that extends from October to April, and a dry season with none or almost no rainfall that extends from May to September (Lopes et al. 2013). The climatological data collected during the period in which the experiments were conducted are shown in Figure 17.

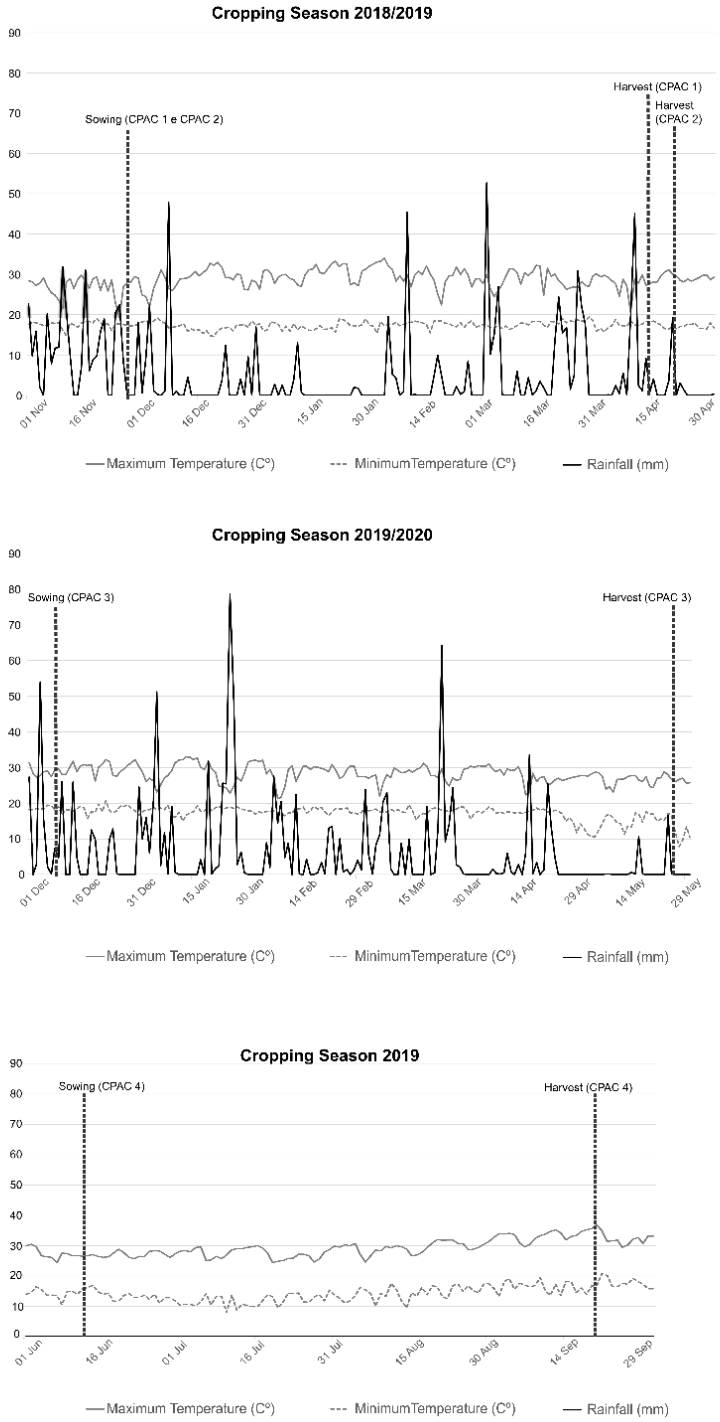


**Figure 17.** Precipitation and maximum and minimum air temperature during experiments CPAC 1 and CPAC 2 (2018/2019), and CPAC 3 (2019)

Before sowing, eight soil samples (0-20 cm layer) were collected from the experimental plots to determine the chemical properties of the soil (Table 9).

**Table 9**. Chemical characteristics of the soils in the experimental area of the Cerrados Agricultural Research Center (CPAC). Soil samples were taken from the 0-20 cm layer and all analyses were performed before sowing.

| Crop season | pH | Al | Ca | Mg | H+Al | K | P | OM | V |
|---|---|---|---|---|---|---|---|---|---|
| | | ---------- $cmol_c.dm^{-3}$ ---------- | | | | -------- $ml.L^{-1}$ -------- | | ------- % ------- | |
| CPAC 1 | 5.99 | 0.03 | 2.15 | 0.84 | 2.74 | 74.00 | 11.00 | 1.66 | 53.83 |
| CPAC 2 | 5.4 | 0.24 | 1.43 | 0.56 | 5.56 | 76.62 | 6.01 | 2.93 | 28.95 |
| CPAC 3 | 5.51 | 0.12 | 1.67 | 0.74 | 5.38 | 144.16 | 18.14 | 2.54 | 34.08 |
| CPAC 4 | 5.9 | 0.03 | 3.22 | 1.43 | 3.78 | 195.62 | 4.66 | 2.48 | 57.82 |

OM; soil organic matter. V; bases saturation = [(K + Ca + Mg)/CEC] × 100. CEC; cation exchange capacity (SB + H + Al).

*Treatments, experimental design, and agronomic analysis*

All experiments were conducted in randomized blocks of seven treatments and six replicates. The plots of the maize trials (CPAC 1, CPAC 2 and CPAC 3) were 5 m (length) x 4 m (width) and consisted of 72 $m^2$ of cultivated area. The spacing between plots was 1 m to avoid cross-contamination between treatments. Experiments CPAC 1 and CPAC 3 received 400 kg of NPK fertilizer (0 20 20) and micronutrients (S, B, Mn, and Zn). Experiment CPAC 2 received 200 kg of NPK fertilizer (0 20 20). After sowing, nitrogen fertilization was applied at 120 kg $ha^{-1}$ N in the control treatment (corresponding to 100% of the recommended dose for the crop) and 90 kg $ha^{-1}$ N (corresponding to 75% of the recommended dose for the crop) in the other treatments. In this fertilizer application, 20 kg $ha^{-1}$ N was applied at the time of sowing and the rest was applied at 30 DAG. Experiments CPAC 1 and CPAC 3 were conducted without irrigation and experiment CPAC 2 was irrigated with a sprinkler system with an average water discharge of 20 mm at 72 h intervals.

In the wheat experiment (CPAC 4), the plots were 5 m (length) x 1 m (width) with 42 $m^2$ of cultivated area. The spacing between plots was 1 m. In wheat, nitrogen fertilizer was applied at 120 kg $ha^{-1}$ N in the control treatment (based on 100% of the recommended dose for the crop) and 60 kg $ha^{-1}$ N (based on 50% of the recommended dose for the crop) in the other treatments in a single dose at 15 DAG. In experiment CPAC 4, a sprinkler irrigation system was used in the same manner as described in experiment CPAC 2.

In both wheat and maize, inoculation with *Azospirillum brasilense* was made one hour before sowing according to the manufacturer's recommendations. The treatments were: (i) control without inoculation with 75% and 50% N for maize and wheat (90 kg $ha^{-1}$ N in maize and 60 kg $ha^{-1}$ N in wheat); (ii) control without inoculation with 100% N (120 kg $ha^{-1}$ N in both crops); (iii) standard inoculation (SI) with *Azospirillum brasilense* Abv5 and Abv6; (iv) SI + ME-LCO; (v) SI + LCOp; (vi) SI + COp and (vii) SI + EPS. SI and SI supplemented with the molecules received 90 kg $ha^{-1}$ N and 60 kg $ha^{-1}$ N, respectively, in the maize and wheat experiments. Nitrogen fertilization was carried out with urea.

During the vegetative phase of growth of wheat (44 DAG), height (cm), stem length (cm), number of shoots, dry biomass and leaf area were evaluated. Agronomic yield components were evaluated when

the plants reached physiological maturity. The following traits were evaluated: plant height (length from the base of the plant to the highest spike), spike length (cm), number of spikes (number of total spikes per 1 $m^2$ of crop area), number of spikelets per spike, and number of grains per spike. After harvest, grain yield (with moisture correction for 13%), total N in grains (TNG), 250 grain mass and hectoliter mass were evaluated. In maize, total grain N in grains (TNG) and grain yield (moisture corrected for 13%) were evaluated after harvest. Total nitrogen content of grains was determined by the modified Bertholot perchloric digestion method (Woolley et al. 1960).

*Statistical analysis*

All analyzes were performed using R software version 3.6.1 (R Core Team 2020). The results were first subjected to a test for normality and homogeneity of the data. After this analysis, the data were subjected to an analysis of variance (ANOVA) and in case of statistical significance, the data were subjected to a Duncan post-hoc test at $p \leq 0.05$. Statistical analyzes were performed using the ExpDes.pt package. Boxplot graphs were created using the ggplot2 package.

**Results**

*Experiments in the greenhouse*

In the experiment with maize, no significant differences were found between treatments for the variables shoot dry weight (SDW), stem height and plant height (Table 10). SI + LCOp and SI + EPS showed an increase in root dry weight (RDW) of 72.2% and 69.0%, respectively, compared to SI. Although there was no statistical difference, an increase in SDW by 59.9%, stem height by 17.4% and plant height by 24.8% was observed in the plants treated with SI + LCOp compared to those treated with SI.

**Table 10.** Shoot dry weight (SDW) and root dry weight (RDW), stem and plant height of maize cultivar DKB 290 VT Pro 3 (Bayer) at 42 DAE, in response to the standard inoculation (SI) with *Azospirillum brasilense* Abv5 and Abv6 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp and exopolysaccharides - EPS). Experiment carried out under greenhouse conditions.

| **Treatments** | **DKB 290** | | | |
|---|---|---|---|---|
| | **SDW (g plant$^{-1}$)** | **RDW (g plant$^{-1}$)** | **Stem height (cm plant$^{-1}$)** | **Plant height (cm plant$^{-1}$)** |
| Non-inoculated control (100% N) | 1.928 | 1.190 $^{ab}$ | 7.610 | 21.462 |
| Standard inoculation (SI) | 1.297 | 0.741 $^{c}$ | 6.228 | 24.050 |
| SI + ME-LCO | 1.582 | 0.885 $^{bc}$ | 6.393 | 27.625 |
| SI + LCOp | 2.075 | 1.276 $^{a}$ | 7.313 | 30.037 |
| SI + COp | 1.996 | 1.081 $^{abc}$ | 6.861 | 28.150 |
| SI + EPS | 1.960 $^{ns}$ | 1.253 $^{a}$ | 6.553 $^{ns}$ | 24.793 $^{ns}$ |
| **Coefficient of Variation (%)** | 25.45 | 24.53 | 17.4 | 16.4 |

All treatments received the equivalent of 90 kg ha$^{-1}$ N. The non-inoculated control received the equivalent of 120 kg ha$^{-1}$ N.
Means (five replicates) followed by the same letter on the same column are not significantly different according to Duncan's test ($p \leq 0.05$).
ns = no statistical significance

Wheat response to the treatments varied among cultivars. There was no statistical difference between the treatments with SI and SI enriched with molecules of the secondary metabolism of *R. tropici*

CIAT 899 when cultivar BRS 394 was evaluated (Table 11). However, cultivar BRS 264 responded to SI with a 32.7% increase in SDW compared to the non-inoculated control. SI + LCOp stood out with an increase in SDW of 22.4% and 62.5% compared to SI and the non-inoculated control, respectively (Table 11). There was no statistical difference between treatments with BRS 264 variety in terms of RDW (Table 11).

**Table 11.** Shoot dry weight (SDW) and root dry weight (RDW) of wheat cultivar BRS 264 and BRS 394 at 34 DAE inoculated with *Azospirillum brasilense* Abv5 and Abv6 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp and exopolysaccharides - EPS). Experiment carried out under greenhouse conditions

| **Treatments** | **BRS 264** | | **BRS 394** | |
|---|---|---|---|---|
| | **SDW (g plant$^{-1}$)** | **RDW (g plant$^{-1}$)** | **SDW (g plant$^{-1}$)** | **RDW (g plant$^{-1}$)** |
| Non-inoculated control (100% N) | 0.342 $^{c}$ | 0.238 | 0.510 | 0.345 |
| Standard inoculation (SI) | 0.454 $^{b}$ | 0.336 | 0.498 | 0.332 |
| SI + ME-LCO | 0.518 $^{ab}$ | 0.362 | 0.592 | 0.386 |
| SI + LCOp | 0.556 $^{a}$ | 0.354 | 0.558 | 0.368 |
| SI + COp | 0.474 $^{ab}$ | 0.356 $^{ns}$ | 0.554 $^{ns}$ | 0.434 $^{ns}$ |
| **Coefficient of Variation (%)** | 16.5 | 24.66 | 15.37 | 22.68 |

All treatments received the equivalent of 60 kg ha$^{-1}$ N. The non-inoculated control received the equivalent of 120 kg ha$^{-1}$ N.
Means (five replicates) followed by the same letter on the same column are not significantly different according to Duncan's test ($p \leq 0.05$).
ns = non significative

*Field experiment*

*Maize*

Three experiments were conducted at the Cerrado Agricultural Research Center (CPAC) experimental plot. In experiment CPAC 1, the addition of the inoculant from *A. brasilense* with LCOp resulted in an average grain yield statistically equal to the control treatment (120 kg ha$^{-1}$ N), which was the best performance obtained with an inoculated treatment (Figure 18). SI + LCOp stood out compared to SI + ME-LCO and SI + COp with increased productivity of 5.71% and 4.9%, respectively (Figure 18). However, LCOp supplementation did not significantly increase productivity compared to SI, SI + EPS and the control (90 kg ha$^{-1}$ N) (Figure 18). In experiment CPAC 2, no statistical difference in grain yield was observed among the evaluated treatments (Figure 18). Similar to experiment CPAC 1, it was also observed in CPAC 3 that the control treatment (120 kg ha$^{-1}$ N) had the highest average grain yield. The other treatments were not statistically different from each other (Figure 18).

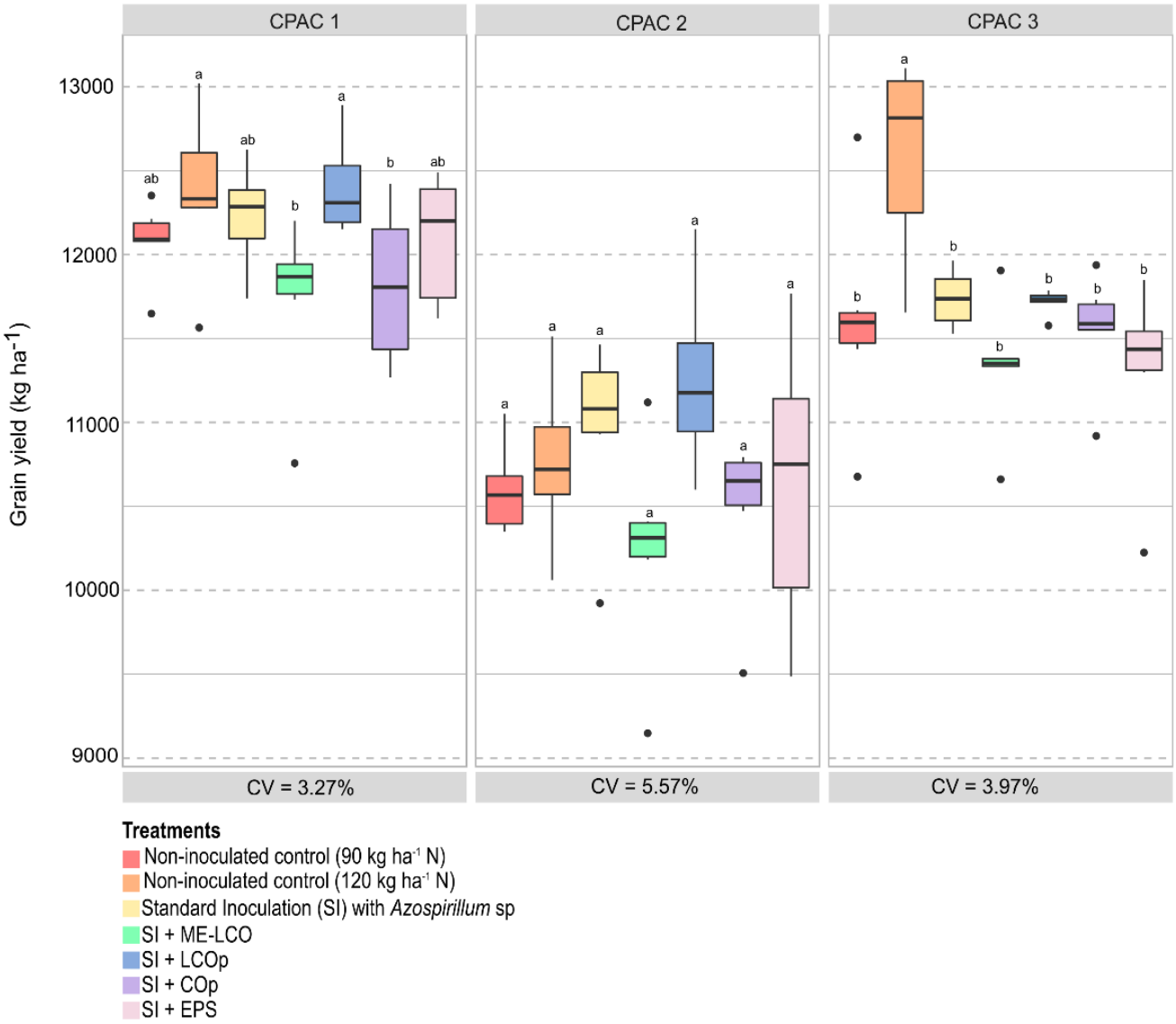


**Figure 18**. Boxplot of grain yield (kg ha$^{-1}$) of CPAC 1 and CPAC 2 experiments conducted with DKB 290 VT Pro (Bayer) Maize-Hybrid and CPAC 3 conducted with the cultivar 30F53VYH (Pioneer). The experiments were located in the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Azospirillum brasilense* Abv5 and Abv6 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides – EPS). Lower and upper box boundaries represent 25th and 75th percentiles, respectively; the line inside the box indicates the median; whiskers on the top and bottom represent the 10th and 90th percentiles, respectively; points above and below the whiskers indicate outliers outside the 10th and 90th percentiles, respectively. Means followed by the same letter are not significantly different according to Duncan's test ($p \leq 0.05$).

The combined analysis of the three experiments, conducted using a similarity matrix, grouped the treatments into two main clusters in the dendrogram (Figure 19). The control treatment without inoculation (90 kg ha$^{-1}$ N) and SI + EPS, SI + ME-LCO, and SI + COp were grouped into a single cluster. Interestingly, the control treatment without inoculation (90 kg ha$^{-1}$ N) and SI + COp showed more similarity to each other, while SI + ME-LCO stood out from the others with a lower average yield than the other treatments (Figure

19). With the highest average grain yield, SI, SI + LCOp and the control treatment (120 kg N $ha^{-1}$) were grouped in a second cluster. SI and SI + LCOp showed greater similarity to each other. The control treatment (20 kg $ha^{-1}$ N), which represents 100% of the recommended dose for the crop, had a higher average grain yield. In general, experiments CPAC 1 and CPAC 3 showed greater similarity in terms of average grain yield.

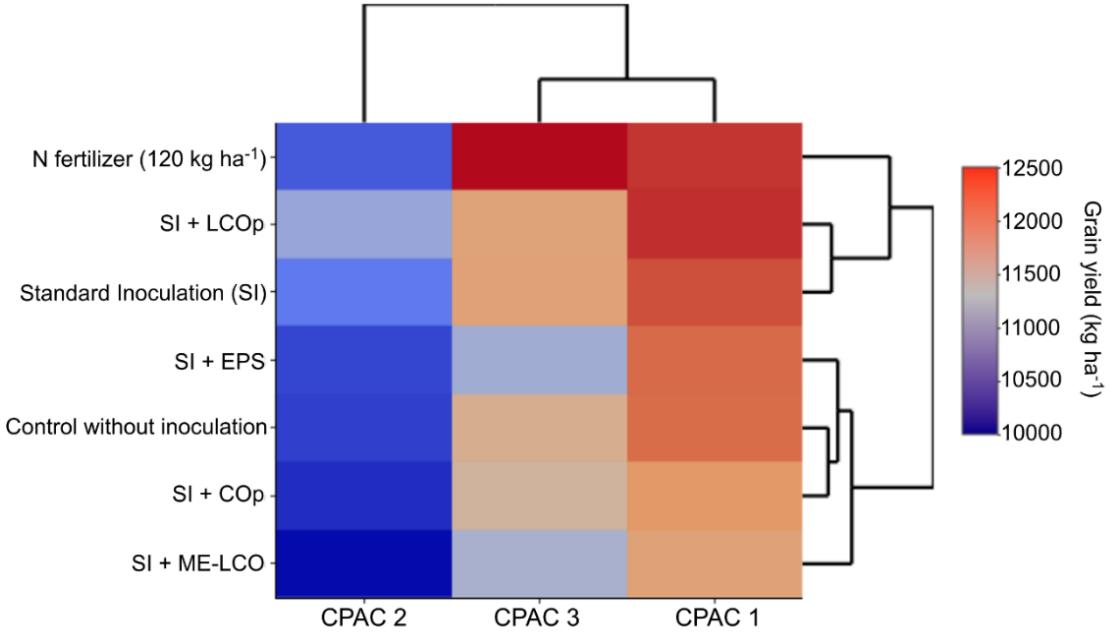


**Figure 19**. Heat map and dendrogram of the Pearson's correlation matrix for the grain yield (kg $ha^{-1}$) of maize in three experiments (CPAC 1 and CPAC 2 in the 2019/2020 crop season and CPAC 3 in the 2020/2021 crop season). The experiments were located in the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Azospirillum brasilense* Abv5 and Abv6 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides – EPS).

Total N in grains (TNG) concentration did not differ between treatments in CPAC 1 and CPAC 3. In CPAC 2, higher TNS was observed in the control treatment (120 kg $ha^{-1}$ N), but this was not statistically different from SI + LCOp and SI + COp (Table 12).

**Table 12**. Total N concentration in grains (TNG) at the V4 growth stage of maize in three experiments (CPAC 1 and CPAC 2 in the 2019/2020 crop and CPAC 3 in the 2020/2021 crop). The experiments were located in the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with *Azospirillum brasilense* Abv5 and Abv6 and its supplementation with molecules of the metabolism of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides – EPS).

| Treatments | CPAC 1 | CPAC 2 | CPAC 3 |
|---|---|---|---|
| | TNG | TNG | TNG |
| | mg $g^{-1}$ | mg $g^{-1}$ | mg $g^{-1}$ |
| Non-inoculated control (90 kg ha $^{-1}$) | 15.19 | 17.53$^{b}$ | 8.21 |
| Non-inoculated control (120 kg ha $^{-1}$) | 15.56 | 19.96$^{a}$ | 8.39 |
| Standard inoculation (SI) with *Azospirillum* sp. | 14.63 | 18.31$^{b}$ | 7.73 |
| SI + ME-LCO | 14.57 | 18.02$^{b}$ | 8.06 |
| SI + LCOp | 14.51 | 18.63$^{ab}$ | 8.12 |
| SI + COp | 15.12 | 18.68$^{ab}$ | 7.84 |
| SI + EPS | 14.69 $^{ns}$ | 18.43$^{b}$ | 7.79 $^{ns}$ |
| **Coefficient of Variation (%)** | **7.14** | **6.17** | **5.84** |

All treatments received the equivalent of 90 kg of N $ha^{-1}$. The non-inoculated control received the equivalent of 120 kg of N $ha^{-1}$

Means (five replicates) followed by the different letters on the same column are significantly different according to Duncan's test ($p \leq 0.05$).

ns = no statistical significance

*Wheat*

Standard inoculation with *A. brasilense* Abv5 and Abv6 and inoculation supplemented with different SMM of *R. tropici* CIAT 899 did not affect the growth of the shoots, which was observed by the height, size, number of spikes and number of spikelets per spike (Table 13). At physiological maturity, higher N concentration in the grains was observed in the control treatment with 120 kg $ha^{-1}$ N compared to the other treatments evaluated (Table 13).

**Table 13.** The yield components evaluated at the harvest were plant height (defined as the distance (cm) from the ground level to the apex of the spike), spike length, number of spikelet, number of grains per spike, total N concentration in the grain (TNG), hectoliter weight and weight of 250 grains of wheat cultivar BRS 264, with or without inoculation with *Azospirillum brasilense* Abv5 and Abv6 (standard inoculation) supplemented with molecules of the secondary metabolite of *Rhizobium tropici* CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides – EPS).

| Treatments | Plant height | Spike length | Spikes | Spikelets per spike | Grains per spike | TNG | Hectoliter weight | Weight of 250 grains |
|---|---|---|---|---|---|---|---|---|
| | ---------- cm plant $^{-1}$ ---------- | | ---------- number plant $^{-1}$ ---------- | | | mg g$^{-1}$ | g | g |
| Non-inoculated control (60 kg ha $^{-1}$) | 57.95 | 6.59 | 195.5 | 15.61 | 43.01 | 21.20$^{b}$ | 84.58 | 8.35 |
| Non-inoculated control (120 kg ha $^{-1}$) | 56.10 | 6.16 | 204 | 14.78 | 36.35 | 25.51$^{a}$ | 84.93 | 8.47 |
| Standard inoculation (SI) | 57.28 | 6.64 | 205 | 15.03 | 39.25 | 22.07$^{b}$ | 84.73 | 8.45 |
| SI + ME-LCO | 58.35 | 6.32 | 196.66 | 15 | 39.43 | 21.61$^{b}$ | 85.38 | 8.09 |
| SI + LCOp | 59.07 | 6.60 | 220.16 | 15.28 | 43.18 | 21.20$^{b}$ | 85.15 | 8.18 |
| SI + COp | 58.35 | 6.39 | 200 | 14.76 | 39.25 | 21.81$^{b}$ | 85.03 | 8.34 |
| SI + EPS | 56.77$^{ns}$ | 6.34$^{ns}$ | 207.33$^{ns}$ | 15.28$^{ns}$ | 38.86$^{ns}$ | 22.53$^{b}$ | 84.40$^{ns}$ | 8.06$^{ns}$ |
| **Coefficient of Variation (%)** | **5.68** | **6.65** | **10.39** | **5.53** | **11.8** | **8.13** | **1.01** | **3.58** |

All treatments received the equivalent of 60 kg of N ha$^{-1}$. The non-inoculated control received the equivalent of 120 kg of N ha$^{-1}$
Parameters determined at physiological maturity
Means followed by the same letter on the same column are not significantly different according to Duncan's test ($p \leq 0.05$)
ns = no statistical significance

Supplementation of the *A. brasilense*-containing inoculant with SMM of *R. tropici* CIAT 899 directly affected the productivity of the crop in CPAC 4 conducted in the field with wheat cultivar BRS 264 (Figure 20). The average grain yield of SI + LCOp was significantly higher than that of the control treatment with 60 kg $ha^{-1}$ N, SI, SI + EMLCO, SI + COp and SI + EPS with an increase of 6.6%, 10%, 7.4%, 18.3% and 8.1%, respectively (Figure 20). However, the control treatment with 120 kg $ha^{-1}$ N stood out from the others, with the average yield reaching 3032 kg $ha^{-1}$, which was 6.8% higher than the yield observed in SI + LCOp (Figure 20). There was no statistical difference between the control treatment with 60 kg $ha^{-1}$ N, SI, SI + EMLCO and SI + EPS (Figure 20). SI + COp showed lower grain yield among the evaluated treatments.

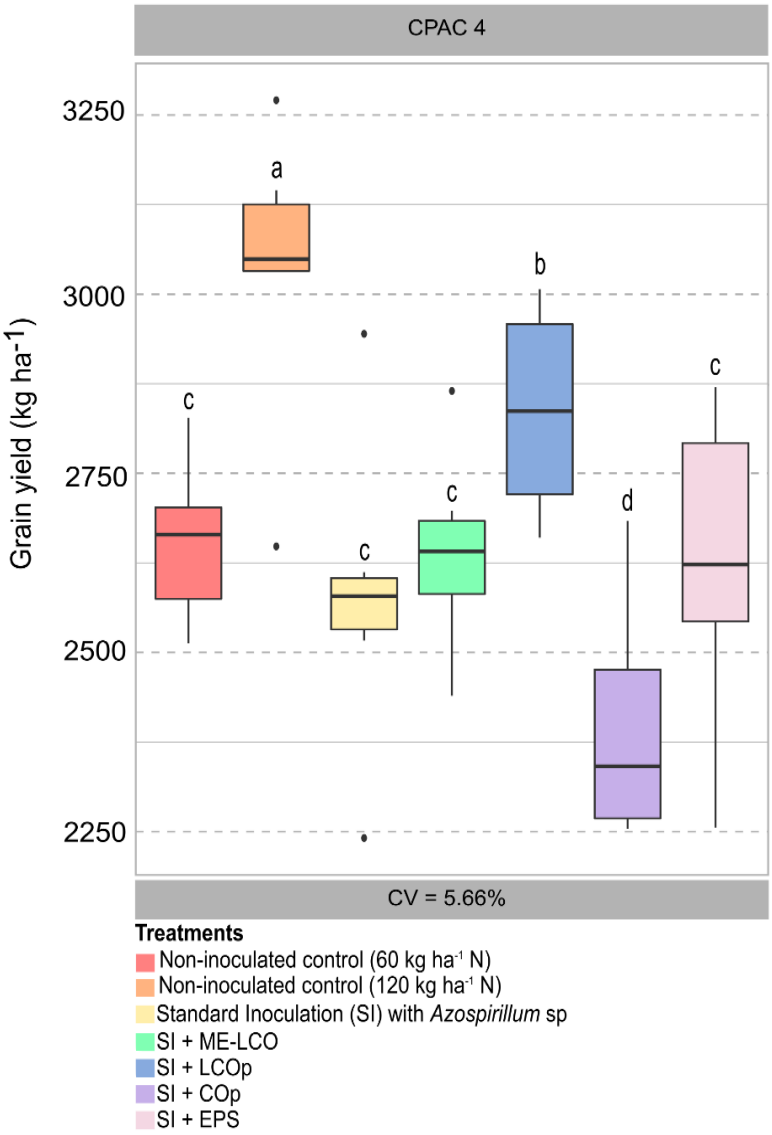


**Figure 20.** Boxplot of grain yield (kg $ha^{-1}$) of CPAC 4 experiment conducted with wheat cultivar BRS 264 RR in a experiments conducted in the 2019 crop seasons located in the experimental field of the Cerrado Agricultural Research Center (Embrapa Cerrados), in response to the standard inoculation (SI) with Azospirillum brasilense Abv5 and Abv6 and its supplementation with molecules of the metabolism of Rhizobium tropici CIAT 899 (metabolic extract containing lipochitoligosaccharides - ME-LCO, purified lipochitoligosaccharides - LCOp, purified chitoligosaccharides - COp, and exopolysaccharides – EPS). Lower and upper box boundaries represent 25th and 75th percentiles, respectively; the line inside the box indicates the median; whiskers on the top and bottom represent the 10th and 90th percentiles, respectively; points above and below the whiskers indicate outliers outside the 10th and 90th percentiles, respectively. Means followed by the same letter are not significantly different according to Duncan's test ($p \leq 0.05$).

**Discussion**

In Brazil, *A. brasilense* strains Abv5 and Abv6 are recommended for the formulation of inoculants for wheat and maize (Hungria et al. 2010). The same strains are also recommended for brachiaria pastures (Hungria et al. 2016). Complete replacement of nitrogen fertilizer use in maize and wheat only by inoculation with *Azospirillum* spp. is an unlikely scenario due to the low contribution of biological nitrogen fixation (BNF) promoted by these bacteria (Fukami et al. 2016; Oliveira et al. 2017). In addition to BNF, *A. brasilense* produces growth regulators (Tien et al. 1979) that support plant uptake of nutrients and water, stimulate root growth, and increase plant nutritional efficiency (Galindo et al. 2019a; Oliveira et al. 2017), resulting in reduced nitrogen fertilizer use and increased grain yield (Hungria et al. 2010; Okon and Labandera-Gonzalez 1994).

In this study, we observed in the greenhouse that the treatment with *A. brasilense* and 90 kg $ha^{-1}$ N (75% of the recommended dose for the crop) did not differ from fertilization with 120 kg $ha^{-1}$ N in terms of SDW, stem diameter and height (Table 10). This highlights that inoculation with *A. brasilense* is a viable strategy to reduce nitrogen fertilization (Fukami et al. 2016). Moreover, the addition of LCOp to the *A. brasilense*-containing inoculant increased root dry weight compared to SI. Purified LCOs at submicromolar concentrations ($10^{-9}$ to $10^{-12}$ M) have biological activity in various plants and act as mitogens that stimulate root growth in non-legume plants (Kidaj et al. 2020; Prithiviraj et al. 2003; Souleimanov et al. 2002). In a study with maize treated with exogenous LCO, Tanaka et al. (2015) observed an increase in lateral root formation compared to plants not treated with LCO. According to Kidaj et al. (2020), several genes responsible for auxin signaling are activated in plants exposed to LCOs, thus, LCOs control the hormone concentration in the plant, directly and indirectly regulating plant growth, especially root growth

In the field, the inoculation of maize enriched with SMM did not show significant differences in grain yield in any of the experiments carried out. These results differ from that reported by other studies that evaluated the combined use of inoculants with bacterial molecules increased maize yield (Marks et al. 2013, 2015), as well as studies showing that the standard inoculation only with A*zospirillum* sp. improves fertilization efficiency in maize (Fukami et al. 2016; García De Salomone and Döbereiner 1996; Okon and Labandera-Gonzalez 1994; Oliveira et al. 2017). According to Galindo et al. (2019), the response of inoculation in grasses is variable, being necessary to consider the interaction of the plant genotype with the bacterial strain, as well as the environmental conditions. There are still several gaps concerning inoculation in grasses due to the variability of results, however, there is a great potential to increase the efficiency of the use of nitrogen fertilizers that can be achieved through inoculation.

The results obtained in the greenhouse with wheat cultivars show that genotypes respond differently to inoculation. It is interesting to note that cultivar BRS 394 did not respond to inoculation, with no statistical difference between the parameters observed. In contrast, cultivar BRS 264 responded positively to inoculation. A significant increase in SDW was observed in SI compared to the control treatment without inoculation. Moreover, SI + LCOp stood out, showing that in this cultivar, in addition to the positive response to inoculation with *A. brasilense*, the addition of LCOp improved the performance of the inoculant when evaluating the SDW. However, Kazi et al. (2016) observed significant increases in root growth, total root volume, size and root surface in wheat inoculated with *A. brasilense*, as Fukami et al. (2016) observed an increase in the root system of the inoculated plants but did not observe increases in SDW in wheat inoculated with *A. brasilense* in a greenhouse. Although, in the present study, no statistical

differences were observed with RDW, it was possible to observe an increase in absolute numbers between the SI treatment compared to the control treatment (120 kg $ha^{-1}$ N).

The suitable response of the wheat BRS 264 to inoculation with *A. brasilense* enriched with SMM observed in the present study corroborates the results of Roque et al. (2021) who observed a superior performance of cultivar BRS 264 treated with inoculants containing diazotrophic bacteria and rhizobia. The results reported by the authors are in agreement with the results of the present study in which we show that the growth promotion and yield gains of BRS 264 wheat are associated with the nodulation factors produced by *R. tropici* CIAT 899 together with the promoter activity of the growth of *A. brasilense*. Additionally, Roque et al. (2021) showed that the gains in productivity and quality of wheat grains are attached to genetic factors inherent to the progeny, and the cultivar BRS 264 showed the best response in the combined use of *A. brasilense* with *R. tropici*. According to Feldmann et al. (2018), the response of wheat cultivars to inoculation is associated with genetic factors.

Inoculation with *A. brasilense* can increase or maintain wheat yield, but with a reduction in the use of nitrogen fertilization (Fukami et al. 2016; Hungria et al. 2010). Although the SI + LCOp treatment stood out in the grain yield of the other treatments supplemented with SMM and SI, the grain yield of this treatment was lower than the treatment that received 120 kg $ha^{-1}$ of N. Inoculation with *A. brasilense* allows a reduction of up to 25% in the proportion of nitrogen fertilization, without yield compromising (Fukami et al. 2016). However, in this study, the N dose was reduced by 50% in the treatments and, due to this, we observed a disparity in grain yield in relation to the treatment that received 100% of the dose. Additionally, in the field experiment with wheat, a 10% increase in grain yield was observed when the standard inoculant was supplemented with LCOp. Possibly, yield gains may be associated with changes in the wheat root system, since no differences were observed in the yield components that evaluate parameters of the leaf area. Changes in the root system of plants caused by *Azospirillum* are the main mechanism of action of the bacteria in promoting plant growth (Boleta et al. 2020; Cassán et al. 2020; Oliveira et al. 2018; Tien et al. 1979). In addition to the effects associated with *A. brasilense*, LCO acts directly on the root and possibly these effects were added to those already expected from the bacteria.

LCOs in non-legume plants can influence root architecture and allow the plant to increase its ability to acquire more nutrients (Rosier et al. 2018). In *Arabidopsis thaliana* (L.) grown in a solution containing 10nM of LCO from *Bradyrhizobium japonicum*, an increase of 33% in the number of initial roots, 33% in root size and 76% in the root surface was observed. (Khan et al. 2011). Interestingly, the authors observed that there was no difference in the shoot and the root treated with CO at concentrations similar to LCO. These results indicate that the presence of N-acyl groups, chain saturations and/or the presence of substituent groups are necessary to generate physiological responses in the plant. In the present study, we observed that supplementation of the standard inoculant with LCOp generated positive responses to growth in wheat and a tendency in maize, reinforcing the hypothesis that LCOp acts promoting plant growth in non-legume plants. Although the phenotypic responses in non-legume plants are not fully elucidated, possibly the LCO chain produced by *R. tropici* CIAT 899 acts positively in plants, activating growth promotion pathways that other molecules do not act.

The results observed in this study with the BRS 264 wheat cultivar are very important for Brazilian agriculture. This cultivar is the most accepted in the Center-West region of Brazil and occupies 70% of the area of wheat cultivation in the region. Its great acceptance is due to its super-precocity combined with high

yields (Caldas 2021). Currently, the cultivar is responsible for the record of daily yield, reaching 160.5 sc $ha^{-1}$ with complete maturation carried out in 100 days. The great acceptance and high profitability of the use of cultivar BRS 264 may turn Brazil self-sufficient in wheat production in the future. Combining the high acceptance of the cultivar with the results obtained by supplementing the inoculant with *A. brasilense* with LCOp may be highly promising to further improve yield results, reduce production costs, increase agricultural sustainability and product acceptance.

The use of SMM to supplement inoculants is still poorly explored but remains a promising strategy for sustainable agriculture. For wheat crops, this is the first study conducted with an *A. brasilense*-based inoculant enriched with such molecules. Positive results have already been obtained for other crops. Moretti et al (2020) observed an 11% increase in grain yield of soybean compared to SI when inoculated with *Bradyrhizobium* spp, *A. brasilense* and microbial metabolites. Marks et al. (2013) obtained 4.8% and 11.4% increase in soybean and maize yield, respectively, with the treatment enriched with bacterial molecules extracted from *B. diazoefficiens* (USDA 110) compared to the standard treatment. Khan et al (2008) observed an increase in photosynthetic rate of maize and soybean treated with LCO and Souleimanov et al (2002) reported an increase in maize and soybean biomass and an increase in root volume with an increase in the length of secondary roots in maize.

Although the mechanism by which LCO acts as a growth promoter in non-host plants is not fully understood, several studies have shown that application of exogenous LCO is associated with growth promotion in non-legumes (Khan et al. 2011; Marks et al. 2013, 2015; Prithiviraj et al. 2003; Schwinghamer et al. 2015b, c; Smith et al. 2015a, b). In legume-rhizobia associations, LCOs function as an important signaling molecule and are a key element for nodule formation. These molecules bind to LysM-type receptors, which, due to their ubiquity, can act as receptors for the LCO molecule in legumes, but also in non-legumes (Rosier et al. 2018). The mechanisms involved in the promotion of plant growth by LCO, beyond nodule formation, may be related to signaling pathways mediated by the LysM receptor, intracellular signaling activated by calcium, or the influence of LCO on the production of growth regulators such as auxin (Rosier et al. 2018). The perception of LCO is now thought to be conserved across a wide range of plant species, although the response to the stimulus varies among species (Souleimanov et al. 2002).

Previous studies with exogenous application of LCO show that this molecule triggers physiological responses in the plant, such as induction of genes involved in the cell cycle, stimulating cell division (Souleimanov et al. 2002), leading to higher germination rate at submicromolar concentrations (Prithiviraj et al. al. 2003), increase in total root size, root surface area, and growth of shoots in non-legume plants (Khan et al. 2011). The results of the present study show that LCO extracted from *R. tropici* CIAT 899 exhibited growth-promoting activity, especially in wheat, improving the performance of the standard inoculant recommended for the crop. Although the study of legume-rhizobia communication molecules in the supplementation of inoculants is incipient, the results presented show that they are potential and promising for use in the field for grasses.

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# Chapter 6 – Factors affecting the host plants response to inoculation with secondary metabolism metabolites of *Rhizobium tropici* CIAT 899: Final discussion

*"Todavia estoy aprendiendo"*

*Francisco de Goya (en un dibujo que hizo a los ochenta años)*

For more than 60 years, the development of products containing beneficial microorganisms has been one of the foci of research in Brazil. Each experiment we conduct brings us one step closer to better understanding the interactions of the microorganisms with the host plants and improve the efficiency of inoculation. Supplementing microbial inoculants with secondary metabolism molecules (SMM) of rhizobia is one of many strategies that have been developed to increase the efficacy and acceptability of the products and to mitigate the harmful effects of mineral fertilizers on the environment, such as reducing the emission of greenhouse gasses.

In this work, the study of promotion of plant growth of agriculturally important crops in Brazil by using recommended commercially available inoculants for crops and supplementing them with SMM of *Rhizobium tropici* CIAT 899 was proposed. Here we corroborate the general hypothesis of this work by showing that supplementation of inoculants with SMM indeed promoted plant growth and increased yield for some crops, and understand that the molecules acted differently among crops.

A number of complex factors appear to be associated with the different results obtained in this work. It was observed that the response to SMM supplementation varied. This could be a consequence of the different factors related to the host, the characteristics of the molecules, the producing microorganism, and the concentration of the molecules. As expected, it is difficult to identify a pattern of behavior of the plants towards the different treatments. Understanding how these factors affect or may affect the efficacy of supplementing inoculants with SMM will help to better understand the action of these molecules and improve the use of this new technology. Therefore, this discussion focuses on the results obtained in this research to provide better insights that may be associated with the responses.

*Source secondary metabolism molecules (SMM) and concentration*

CIAT 899 is one of the bacteria recommended for common bean inoculation in Brazil because of its high stability of the plasmid containing the genes for nodulation and biological nitrogen fixation, high tolerance to environmental stress conditions, such as survival in acidic soils (del Cerro et al., 2015; Hungria et al., 2000; Hungria; Campo; Mendes, 2003; Ormeño-Orrillo et al., 2012). One of the most impressive features of the strain is the production of a variety of nodulation factors under salt stress, high temperature, and acidity, and in the presence of the inducing flavonoids. (Jiménez-Guerrero et al. 2018).

Nodulation factors are produced by rhizobia via transcriptional factors (NodD proteins) that are constitutively expressed and activate the production of LCOs (del Cerro et al. 2017). Previous studies with

CIAT 899 show that this strain has five different nodD genes in the symbiotic plasmid (Ormeño-Orrillo et al. 2012) and possibly each of these nodD proteins responds to different environmental factors, such as salt stress, for the production of LCOs (del Cerro et al. 2017). Therefore, the production of nodulation factors even in the absence of flavonoid-like inducers makes CIAT 899 an interesting strain for the production of secondary metabolites (Marks et al. 2015).

This work aimed to develop a formulation with the potential to be commercialized. Thus, the aim was to produce metabolites with a maximum reduction in production costs so that the final product could provide a good cost-benefit ratio for both the industry and the producer. CIAT 899 is not only a bacterium with a fully elucidated genome, but also known to produce nodulation factors in the absence of the inducing flavonoid, in this case, apigenin. With regard to the industrial sector, the reduction of expenses for the inducer is interesting, since its use weighs on the production costs of molecules.

In addition, CIAT 899 produces a wide range of nodulation factors (del Cerro et al., 2016), making it a versatile strain capable of promoting nodulation in a wide range of legumes. This factor was considered when selecting CIAT 899 as a source for SMM extraction, as the strain molecules were expected to have high biological activity for a wide range of plants. CIAT 899 was also selected as a source of SMM because of its rapid growth in the culture medium, an important characteristic of microorganisms for industrial use. Moreover, this strain produces EPS with great efficiency in a culture medium rich in carbon sources and low in nitrogen (Castellane et al. 2017).

Besides the microorganism producing the SMM, another factor may be related to the final result is the concentration of the molecules. At submicromolar concentrations, LCOs induce physiological responses in the host plant (Prithiviraj et al. 2002). In this work, it was found that concentrations up to 2µl $ml^{-1}$ of purified CO induced the formation of nodular primordia in soybean and common bean (Chapters 1 and 3), confirming the findings of Khan et al. (2011). The concentration of molecules used in this work was proposed following the results presented by Marks et al. (2013, 2015) using 1 µl $ml^{-1}$ ME-LCO from CIAT 899 in soybean and maize. The concentration of LCO was set to half the concentration of ME-LCO because the purified LCO has highly biological activity. The concentration of CO was determined from the results obtained by the formation of nodular primordia.

It is possible that the concentration of the molecules was a determining factor in the host response. As mentioned earlier, it was expected that the crops would not respond the same or even similar to different molecules. Since this work is pioneering in the study of LCO and other molecules for a variety of cultures, we chose to first determine standard concentrations of molecules and dose adjust in further studies.

*Interaction between host plant and SMM.*

Soybean, maize, wheat, and common bean responded differently to standard inoculation (SI) enriched with SMM. The interaction between the host plant and SMM in terms of the observed parameters supports the hypothesis that the molecules would generate physiological responses in the plants, albeit in different ways. In soybean and wheat, productivity increases and growth-promoting effects were clear when supplemented with SMM in the inoculants recommended for each crop. However, in maize and common bean, no difference was found between the effects observed only in the treatment with SI. Figure 21 schematic graphic that summary the results presented in this work, showing how the different crops responded to inoculation supplemented with SMM.

Grasses responded positively to inoculation with *A. brasilense* with purified LCO, while soybeans showed positive results to inoculation with *Bradyrhizobium* spp. supplementation with ME-LCO. These results confirm previous studies showing that LCO promotes growth, lateral root development, and increased nutrient uptake in grasses in a non legumes (Smith et al. 2015). These results were already expected in grasses, as several studies show that LCO has hormone-like effects in this type of plant and promotes plant growth in a non-specific manner (Zhang and Smith 2002).

From a different perspective, inoculation with purified LCO in soybean did not alter plant response, possibly due to the specificity of the host's symbiotic relationship with the rhizobia, which is determined by the molecular structure of LCO (Lerouge et al. 1990; López-Lara et al. 1995). CIAT 899 is not the microsymbiont of soybean because the LCO structure of CIAT 899 consists of a typical linear structure comprising a chain of three to five N-acetyl-D-glucosamine (GlcNAc) residues with N-methyl or sulfated substituents on the reducing or nonreducing GlcNAc residues, respectively (del Cerro et al., 2019). This molecular structure differs from the structure recognized by soybean produced by its microsymbiont *Bradyrhizobium* spp. which consists of molecules containing substituent groups formed by methyl fucose (Mabood et al. 2006).

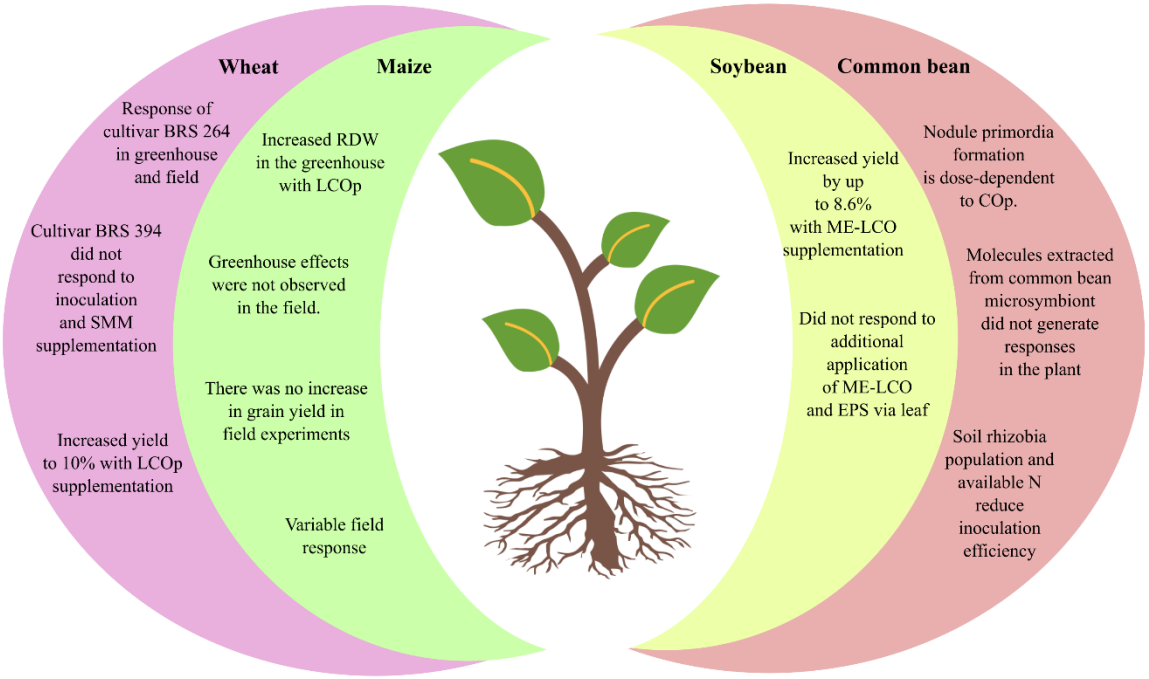


**Figure 21.** Schematic graphic representing the response of wheat, maize, soybean and common bean with the inoculation with the respective inoculant supplemented with metabolic extract containing lipochitoligosaccharides (ME-LCO), purified lipochitoligosaccharides (LCOp), purified chitoligosaccharides (COp) and exopolysaccharides (EPS) extracted from *Rhizobium tropici* CIAT 899.

On the other hand, inoculation with ME-LCO promoted soybean growth and increased grain yield in the field (Chapters 2 and 3). We believe that soybean responded positively to inoculant supplementation with this molecule not only because it contains LCO in its composition, but also because of its interaction with other SMMs present such as auxins, flavonoids, EPS and CO. Zhang and Smith (1995) observed that direct application of flavonoids to the rhizospheric region of inoculated soybean increased the grain yield

of the plant. Moreover, Feng et al. (2019) showed that the combination of LCO and CO acted synergistically to increase symbiotic signaling and suppress plant immunity during the establishment of mycorrhizal colonization.

Interestingly, supplementation with ME-LCO via seed in soybean was beneficial in experiments conducted in different seasons and stood out from other treatments, including additional application via foliar. This way, we demonstrated that inoculant supplementation with ME-LCO of CIAT 899 could be interesting for new formulations.

In another scenario, supplementation with the molecules did not favor bean growth in the field. In contrast to the results obtained with soybean, no increase in yield and parameters related to plant growth was observed in the field in common bean with SMM supplementation. We believe that this is due to difficulties already reported in the literature that reduce the effectiveness of biological nitrogen fixation in the plant, such as the presence of rhizobia and the nitrogen available in the soil (Vargas et al. 2000).

*Final considerations*

The results presented in this paper shed light on a new generation of inoculants. We demonstrated that SMM has physiological effects on legumes (soybean) and non-leguminous (wheat) and that its use in conjunction with the standard inoculant can enrich the product. This is the first work to investigate the growth-promoting effects of the molecules studied in common bean and wheat, and to show the role of CO in nodule primordia formation in common bean and soybean. We hope that this work will contribute to Brazilian agriculture in the study of new sustainable and biotechnological solutions.

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