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Co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* in the Soybean Crop

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Abstract

The objective of this paper was to evaluate the effect of the co-inoculation of *Bradyrhizobium japonicum* (*B. japonicum*) and *Azospirillum brasilense* (*A. brasilense*) on the agronomic characteristics of the soybean crop in the central region of State of Rio Grande do Sul (RS). The experiment was conducted during three harvests (2016/2017, 2017/2018, 2018/2019), in the central region of the State of RS. The treatments were: T1-inoculation with 1 dose B. *japonicum*; T2-co-inoculation with 1 dose of *B. japonicum* + 1 dose of *A. brasilense*; T3-co-inoculation with 2 doses of *B. japonicum* + 1 dose of *A. brasilense*; T4-co-inoculation with 2 doses of *B. japonicum* + 2 doses of *A. brasilense*; T5-co-inoculation with 1 dose of *B. japonicum* + 2 doses of *A. brasilense*; T6-without inoculation (control). The determinations were: shoot and root system dry mass, number and dry mass of nodules, and grain yield. It was observed that the use of co-inoculation with 1 dose of *B. japonicum* + 2 doses of *A. brasilense* provided a higher dry mass of nodules and grain yield of the soybean crop.

Keywords: biological nitrogen fixation, Inoculation, *Glycine max*

1. Introduction

The soybean crop (*Glycine max* L.) is one of the most cultivated crops in Brazil and the world, and Brazil is one of the largest producers of this oilseed. It consists of a relevant source of plant protein, both for human and animal food, besides being a source of vegetable oil for the feeding and production of biofuels (Seixas et al., 2020). The crop has a high demand for nitrogen (N), requiring 80 kg of N for the production of 1000 kg of grains (Hungria et al., 2001). The high costs of chemical nitrogen fertilizers practically make it impossible to use these to meet the needs of the crop, so biological nitrogen fixation (BNF) is a fundamental technology for the viability of soybean crops (Hungria et al., 2001).

The BNF in soybean crop is performed by bacteria of the genus *Bradyrhizobium*, which establish a symbiotic relationship with the plant through the root system, developing nodules that consist of plant cells infected with these bacteria capable of reducing atmospheric nitrogen to a shape assimilated by the plant (Hungria et al., 2001). The amount of N supplied via BNF to soybean crops can supply any crop need for certain productive ceilings. However, the emergence of soybean cultivars with high productive ceilings, combined with research results in which soybean has responded to N application, again generated doubts about the need to fertilize soybean with nitrogen fertilizers (Moreno et al., 2018). With this the search for new technologies, to meet the needs of N of culture becomes necessary.

In this sense, the co-inoculation technique emerges as an alternative for the soybean crop. This technique advocates uniting bacteria of the genus *Brayrhizobium* that through BNF contribute to the supply of N to the crop, with bacteria of the genus *Azospirillum* whose benefits come mainly from the production of phytomoniums that promote plant growth, mainly from the root system (Prando et al., 2019). As a result, soybean plants co-infected with *Bradyrhizobium* and *Azospirillum* have a more abundant and early nodulation (Chibeba et al., 2015; Hungria et al., 2015), with an average productivity gain of 16% (Hungria et al., 2013), which is twice that provided by the annual inoculation only with *Bradyrhizobium*.

The objective of this study was to evaluate the effect of the co-inoculation of *B. japonicum and A. brasilense* on the agronomic characteristics of the soybean crop in the central region of Rio Grande do Sul state.

2. Material and methods

The research was conducted during three agricultural seasons, 2016/2017, 2017/2018 and 2018/2019 in irrigated area under central pivot at the Federal Institute Farroupilha Campus São Vicente do Sul, located in the central Depression region of the State of Rio Grande do Sul (Latitude: -29,691667 Longitude: -54,679445). At 129 meters of average altitude, the climate of the place according to Köppen is classified as a humid temperate climate with hot summer (Cfa) and the soil classified as Ultisols.

The local soil has been cultivated with grain crops for more than 50 years and for at least 10 years it is carried out under no-tillage system. According to the recommendations of the Soil Fertility and Chemistry Commission (2016), for grain crops, the pH, phosphorus (P) content in layer 0-10, Calcium and Magnesium contents are adequate (Table 1). Organic matter, P content in layer 10-20 and potassium levels are low (Table 1).

Table 1. Soil chemical characteristics of the study site

Prof.	Ca	Mg	CEC	pH	OM	m	BS	Argila	P	K
------ m -----	---------- cmolc dm^{-3} ---------				--------------------- % ---------------------				---- mg dm^{-3} ----	
0-0.1	4.5	1.3	5.7	5.5	1.9	0.0	40.6	12	34	56
0.1-0.2	3.9	1.1	5.5	5.4	1.2	7.3	50.9	15	14	34

Note. Ca-calcium; Mg-magnesium; CEC-cation exchange capacity; OM-organic matter; m-aluminum saturation; BS-base saturation; P-phosphorus; K-potassium.

The experimental design was randomized blocks containing 6 treatments with 4 replications. The treatments consisted of co-inoculation of soybean seeds with *B. japonicum* and co-inoculation of soybean seeds with *B. japonicum* and *A. brasilense*, as described: T1-inoculation with 1 dose *B. japonicum*; T2-co-inoculation with 1 dose of *B. japonicum* + 1 dose of *A. brasilense*; T3-co-inoculation with 2 doses of B. *japonicum* + 1 dose of *A. brasilense*; T4-co-inoculation with 2 doses of *B. japonicum* + 2 doses of *A. brasilense*; T5-co-inoculation with 1 dose of *B. japonicum* + 2 doses of *A. brasilense*; T6-without inoculation (control).

The commercial product Masterfix® at a concentration of 5×10^9 and a dosage of 100 ml for every 50 kg of seeds as equivalent to 1 dose of *B. japonicum* was used as a source of 5×10^9 and a dosage of 100 ml for every 50 kg of seeds as equivalent to 1 dose of *B. japonicum.* The source of *A. brasilense* was the commercial product Biomax Azum®, at a concentration of $3x10^9$, being the dose and 100 ml for every 20 kg of seeds as equivalent to 1 dose of *A. brasilense*.

For the implementation of the research, soil collection was performed for chemical and physical analysis of the soil. From the soil analysis, soil acidity correction was performed and fertilization was also defined for the crop following the recommendations of the Fertilization and Liming Manual for the states of Rio Grande do Sul and Santa Catarina (Soil Chemistry and Fertility Commission, 2016). The seed was inoculated on the day of sowing, where it was performed respecting the agroclimatic zoning for the crop, being performed with spacing between lines of 0.45 m.

In the 2016/2017 agricultural year, sowing was carried out on November 23, 2016, and cultivar DM 61i 59 RSF IPRO was used. In the agricultural year 2017/2018, the cultivar used was the TMG 7062 IPRO, with sowing on November 29, 2017. And, in the agricultural year 2018/2019, sowing was carried out on November 29, 2018, using the cultivar TMG 703 IPRO. The three experiments were carried out in the same field, however, in different locations in the field each year of implantation to avoid the influence of the population of bacteria from one experiment to the next.

The seeds were treated with fungicide and insecticide before sowing with, pyraclostrobin, methyl thiophanate, and fipronil at dosages recommended by the manufacturers. Crop management concerning the control of invasive plants, pests, and diseases was performed when necessary following the technical recommendations for the soybean crop.

The variables evaluated were: number of nodules per plant, dry mass of nodules per plant, dry mass of root, dry mass of shoots, and crop yield. For the evaluation of the variables number and dry mass of nodules, root dry mass, and shoots, ten plants were randomly collected in each experimental unit.

The number of nodules per plant was evaluated by collecting the plants at stage R1, and the roots were washed and nodules manually detached from the roots to count the number of nodules per plant. After the nodules, they were submitted to drying, in a forced-air oven, at a constant temperature of 60 °C until the constant mass was obtained, at which time they were weighed with a precision scale to obtain the mass of nodules.

With the nodules removed, the root system of the plants was dried in a forced-air oven at 60 °C until a constant mass measured on a precision scale was obtained. The dry mass of the aerial part of the plants was determined by separating the aerial part and drying it in an air oven forced to 60 °C to constant mass, and after being subjected to precision scale weighing.

To determine the productivity, the plants were harvested in a useful area of 4 m² of each experimental unit. The vegetables were threshed, and the grains cleaned and dried. After that, grain moisture was corrected at 13% and the estimated yield in kg ha^{-1}. The data were submitted to analysis of variance and the means compared by the Tukey test at 5% probability of error, with the aid of the SISVAR package (Ferreira, 2011).

3. Results and Discussion

There was no significant interaction between the variables studied and the years in which the study was conducted. For the variables root dry mass and shoot dry mass, no significant differences were observed between the treatments studied (Table 2). Similar results were obtained by Bárbaro et al. (2009) and Zuffo et al. (2015). However, Braccini et al. (2016) and Bulegon et al. (2016) observed results contrasting with those of the present study for these variables. According to Bulegon et al. (2016), the co-inoculation response in the increase of shoot mass can be influenced by the cultivar used, which may explain the results obtained in the present study.

Table 2. The dry mass of roots and aerial part of soybean plants submitted inoculation with *Bradyrhizobium japonicum* (*B. japonicum*) and co-inoculation with *B. japonicum* and *Azospirillum brasilense* (*A. brasilense*) in the agricultural years 2016/2017, 2017/2018, 2018/2019

Treatments	Root dry mass (g)			Shoot dry mass (g)		
	2016/17	2017/18	2018/19	2016/17	2017/18	2018/19
Inoculation (*B. japonicum*)	2.93	2.92	2.99	26.25	25.14	26.43
Co-inoculation (1 dose de *B. japonicum* + 1 dose *A. brasilense*)	3.24	3.12	3.19	30.51	29.96	30.21
Co-inoculation (2 doses *B. japonicum* + 1 dose *A. brasilense*)	3.24	3.30	3.27	28.73	28.65	29.76
Co-inoculation (2 doses *B. japonicum* + 2 doses *A. brasilense*)	3.40	3.28	3.33	34.43	32.50	33.65
Co-inoculation (1 doses *B. japonicum* + 2 doses *A. brasilense*)	3.68	3.70	3.55	30.85	33.67	32.87
No inoculation	2.97	2.84	2.99	25.91	24.11	25.76
Test F	2.32^{ns}	2.34^{ns}	2.24^{ns}	1.44^{ns}	1.39^{ns}	1.42^{ns}
CV	12.6	16.7	14.5	20.2	20.9	19.8

The number of nodules per plant (Figure 1) increased with the use of inoculation and co-inoculation when compared to treatment without inoculation.

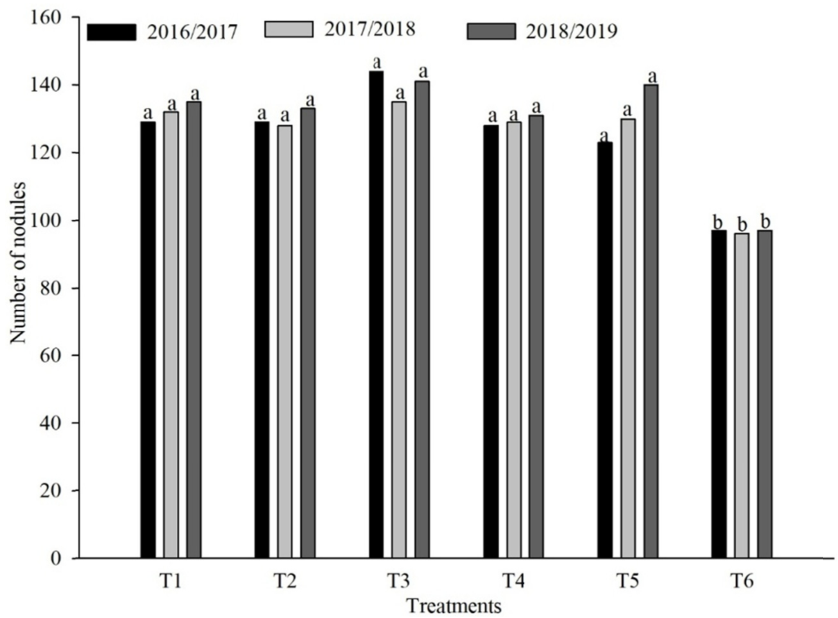

Figure 1. Number of nodules per plant in soybean crop inoculated with *B. japonicum* and co-inoculated with *B. japonicum* and *A. brasilense* in the agricultural years 2016/2017, 2017/2018 and 2018/2019. São Vicente do Sul, 2020

Note. T1-inoculation with 1 dose *B. japonicum*; T2-co-inoculation with 1 dose of *B. japonicum* + 1 dose of *A. brasilense*; T3-co-inoculation with 2 doses of *B. japonicum* + 1 dose of *A. brasilense*; T4-co-inoculation with 2 doses of *B. japonicum* + 2 doses of *A. brasilense*; T5-co-inoculation with 1 dose of *B. japonicum* + 2 doses of *A. brasilense*; T6-without inoculation.

A greater presence of nodules in plants with the use of inoculation or co-inoculation was also observed in several studies, including Bárbaro et al. (2009), Hungria et al. (2013), Chibeba et al. (2015) and Braccini et al. (2016). This is an expected and the desired result with the use of the practice, already consolidated, of inoculation, and is due to the largest population of *B. japonicum* bacteria that was added to the seeds. Besides et al. (2010) state that bacteria of the genus *Azzospirillum* cause alterations in the morphology of the root system resulting in more nodulation-capable root sites, highlighting the contribution of co-inoculation in this aspect.

Co-inoculation with 1 dose of *B. japonicum* + 2 doses of *A. brasilense* (T5) resulted in a higher dry mass of the nodules in the three years of the study (figure 2).

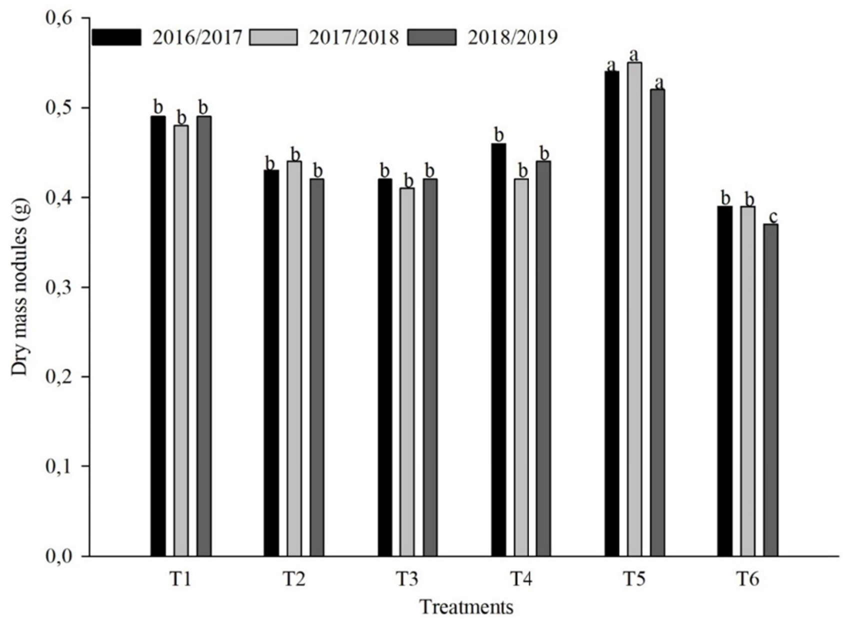

Figure 2. The dry mass of nodules per plant in soybean crop inoculated with *B. japonicum* and co-inoculated with *B. japonicum* and *A. brasilense* in the agricultural years 2016/2017, 2017/2018 and 2018/2019. São Vicente do Sul, 2020

Note. T1-inoculation with 1 dose *B. japonicum*; T2-co-inoculation with 1 dose of *B. japonicum* + 1 dose of *A. brasilense*; T3-co-inoculation with 2 doses of *B. japonicum* + 1 dose of *A. brasilense*; T4-co-inoculation with 2 doses of *B. japonicum* + 2 doses of *A. brasilense*; T5-co-inoculation with 1 dose of *B. japonicum* + 2 doses of *A. brasilense*; T6-without inoculation.

Similar results were obtained by Benintende et al. (2010), Chibeba et al. (2015), and Braccini et al. (2016). The use of co-inoculation in legumes results in greater nodulation in the root crown (Yahalom, Okon, & Dovrat, 1990) since A. brasilense induces early nodulation (Chibeba et al., 2015) because the nodules present in the root crown are the first to form and are associated with strains inoculated with *rhizobium* (Cardoso et al., 2009). Thus, it can have better-formed nodules and of higher mass, therefore, more efficient in the biological fixation of N.

Moreover, the early formation of nodules is very important for the establishment of symbiosis and the beginning of the N fixation process, especially for short-cycle crops such as soybean (Chibeba et al., 2015).

As observed for the dry mass of nodules, the grain yield of the soybean crop was also higher in the treatment in which the co-inoculation of 1 dose of *B. japonicum* + 2 doses of *A. brasilense* was used in the three years of the study (Figure 3). Increases in grain yield with the use of co-inoculation were also reported by Bárbaro et al. (2009), Hungria et al. (2015), Bulegon et al. (2016), Gitti (2016), Braccini et al. (2016), and Naoe et al. (2020).

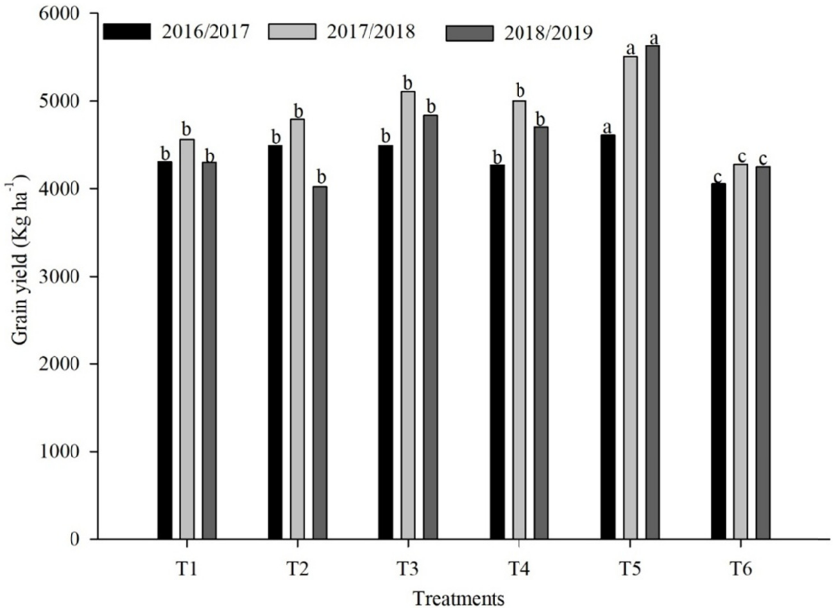

Figure 3. Grain yield of soybean crop submitted to inoculation and co-inoculation with *B. japonicum* and *A. brasilense* in the agricultural years 2016/2017, 2017/2018, and 2018/2019.

Note. T1-inoculation with 1 dose *B. japonicum*; T2-co-inoculation with 1 dose of *B. japonicum* + 1 dose of *A. brasilense*; T3-co-inoculation with 2 doses of *B. japonicum* + 1 dose of *A. brasilense*; T4-co-inoculation with 2 doses of *B. japonicum* + 2 doses of *A. brasilense*; T5-co-inoculation with 1 dose of *B. japonicum* + 2 doses of *A. brasilense*; T6-without inoculation.

These results were attributed to the combined effect of the higher growth of the root system obtained with the use of *A. brasilense* (Prando et al., 2019), which results in plants with abundant nodulation (Hungria et al., 2015) and consequently better supplied in N, due to BNF of the bacterium *B. japonicum*. The greatest resistance to water deficit is also highlighted (Hungria et al., 2015; Naoe et al., 2020), observed with the use of co-inoculation in the soybean crop.

The results obtained in the three years of conduction of this study allow us to affirm that for the region of the Central Depression of the State of Rio Grande do Sul, the co-inoculation with 1 dose of *B. japonicum* and 2 doses of *A. brasilense* is the best combination of doses of these bacteria for use in soybean. In this sense, Hungria et al. (2013) observed that very high doses of A. brasilense did not result in benefits for the culture, because, according to Bhattacharyya and Jha (2012) these bacteria produce some compounds and hormones not yet fully known that can inhibit the plant growth.

4. Conclusion

The co-inoculation of *B. japonicum* and *A. brasilense* resulted in a higher mass of nodules and grain yield of the soybean crop in the three years of study.

Under the conditions of this study, the combination of 1 dose of *B. japonicum* and 2 doses of *A. brasilense* presented the best results for the soybean crop.

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References

Bárbaro, I. M., Machado, P. C., Junior, L. S. B., Ticelli, M., Miguel, F. B., & Da Silva, J. A. A. (2009). Produtividade da soja em resposta a inoculação padrão e co-inoculação. *Colloquium Agrariae, 5*(1), 01-07. https://doi.org/10.5747/ca.2009.v05.n1.a0040

Benintende, S., Uhrich, W., Herrera, M., Gangge, F., Sterren, M., & Benintende, M. (2010). Comparison of coinoculation with *Bradyrhizobium japonicum* and *Azospirillum brasilense* with inoculation with

Bradyrhizobium japonicum on nodulation, growth, and N accumulation in the soybean crop. *Agriscientia, 27*(2), 71-77.

Bhattacharyya, P., & Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. *World Journal of Microbiology & Biotechnology, 28*(4), 1327-1350. https://doi.org/10.1007/s11274-011-0979-9

Braccini, A. L., Mariucci, G. E. G., Suzukawa, A. K., Lima, L. H. S., & Piccinin, G. G. (2016). Coinoculação e modos de aplicação de *Bradyrhizobium japonicum e Azospirillum brasilense* e adubação nitrogenada na nodulação das plantas e rendimento da cultura da soja. *Scientia Agraria Paranaensis, 15*(1), 27-35. https://doi.org/10.18188/1983-1471/sap.v15n1p27-35

Bulegon, L. G., Rampim, L., Klein, J., Guimarães, V. F., Battistus, A. G., & Inagaki, A. M. (2016). Componentes de produção e produtividade da cultura da soja submetida a inoculação de *Bradyrhizobium* e *Azospirillum. Terra Latinoamericana, 34*(2), 169-176. Retrieved from http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0187-57792016000200169&lng=es&nrm=iso

Cardoso, J. D., Gomes, D. F., Goes, K. C., Fonseca, N. S., Dorigo, O. F., Hungria, M., & Andrade, D. S. (2009). Relationship between total nodulation and nodulation at the root crown of peanut, soybean and common bean plants. *Soil Biology and Biochemistry, 41*(8), 1760-1763. https://doi.org/10.1016/j.soilbio.2009.05.008

Chibeba, A. M., Guimaraes, M. F., Brito, O. R., Nogueira, M. A., Araujo, R. S., & Hungria, M. (2015). Co-inoculation of soybean with *Bradyrhizobium* and *Azospirillum* promotes early nodulation. *American Journal of Plant Sciences, 6*, 1641-1649. https://doi.org/10.4236/ajps.2015.610164

Comissao de Química e Fertilidade do Solo-RS/SC. (2016). *Manual de adubação e calagem para os estados do Rio Grande do Sul e Santa Catarina*. Núcleo Regional Sul-Sociedade Brasileira de Ciência do Solo.

Ferreira, D. F. (2011). Sisvar: A computer statistical analysis system. *Ciência e Agrotecnologia, 35*(6), 1039-1042. https://doi.org/10.1590/S1413-70542011000600001

Gitti, D. C. (2016). Inoculação e coinoculação na cultura da soja. *Tecnologia e produção: Soja 2014/2015* (Vol. 01, pp. 01-23). Retrieved from https://www.fundacaoms.org.br/base/www/fundacaoms.org.br/media/attachments/234/234/newarchive-234.pdf

Hungria, M., Campo, R. J., & Mendes, I. C. (2001). Fixação biológica do nitrogênio na cultura da soja. *Circular Técnica Embrapa Soja, 35*, 1-48. Retrieved from https://ainfo.cnptia.embrapa.br/digital/bitstream/CNPSO/18515/1/circTec35.pdf

Hungria, M., Nogueira, M. A., & Araujo, R. S. (2013). Co-inoculation of soybeans and common beans with Rhizobia and Azospirilla: Strategies to improve sustainability. *Biology and Fertility of Soils, 49*, 791-801. https://doi.org/10.1007/s00374-012-0771-5

Hungria, M., Nogueira, M. A., & Araujo, R. S. (2015). Soybean seed co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*: A new biotechnological tool to improve yield and sustainability. *American Journal of Plant Sciences, 6*(6), 811-817. https://doi.org/10.4236/ajps.2015.66087

Moreno, G., Albrecht, A. J. P., Pierozan Junior, C., Pivetta, A. T., Tessele, A., Lorenzetti, J. B., & Furtado, R. C. N. (2018). Application of nitrogen fertilizer in high-demand stages of soybean and its effects on yield performance. *Australian Journal of Crop Science, 12*(1), 16-21. https://doi.org/10.21475/ajcs.18.12.01.pne507

Naoe, A. M. de L., Peluzio, J. M., Campos, L. J. M., Naoe, L. K., & Silva, R. A. (2020). Co-inoculation with *Azospirillum brasilense* in soybean cultivars subjected to water déficit. *Revista Brasileira de Engenharia Agrícola e Ambiental, 24*(2), 89-94. https://doi.org/10.1590/1807-1929/agriambi.v24n2p89-94

Prando, A. M., Oliveira, A. B., Lima, D., Possamai, E. J., Reis, E. A., Nogueira, M. A., ... Conte, O. (2019). Coinoculação da soja com *Bradyrhizobium* e *Azospirillum* na safra 2018/2019 no Paraná. *Circular Técnica Embrapa, 156*, 1-20. Retrieved from https://www.infoteca.cnptia.embrapa.br/infoteca/bitstream/doc/1117312/1/Circtec156.pdf

Saikia, S. P., Dutta, S. P., Goswami, A., Bhau, B. S., & Kanjilal, P. B. (2010) Role of Azospirillum in the improvement of legumes. In M. S. Khan, A. Zaidi, & J. Musarrat (Eds.), *Microbes for Legume Improvement* (pp. 389-408). Viena, Austria: Springer. https://doi.org/10.1007/978-3-211-99753-6_16

Seixas C. D. S., Neumaier, N., Junior, A. A. B., Krzyzanowski, F. C., & Leite, R. M. V. B. (2020). *Tecnologias de produção de soj*a. Sistemas de Produção Embrapa. Retrieved from https://ainfo.cnptia.embrapa.br/digital/bitstream/item/214690/1/SP-17-2020-online.pdf

Yahalom, E., Okon, Y., & Dovrat, A. (1990). Possible mode of action of *Azospirillum brasilense* strain Cd on the root morphology and nodule formation in burr medic (*Medicago polymorpha*). *Canadian Journal of Microbiology, 36*, 10-14. https://doi.org/10.1139/m90-003

Zuffo, A. M., Rezende, P. M., Bruzi, A. T., Oliveira, N. T., Soares, I. O., Neto, G. F. G., … Silva, L. O. (2015). Coinoculação de *Bradyrhizobium japonicum* e *Azospirillum brasilense* na cultura da soja. *Revista de Ciências Agrarias, 38*(1), 87-93. Retrieved from http://www.scielo.mec.pt/scielo.php?script=sci_arttext&pid=S0871-018X2015000100013&lng=pt&nrm=iso