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LUIZ GUSTAVO MORETTI DE SOUZA

BIOTECHNOLOGICAL POTENTIAL OF GROWTH-PROMOTING BACTERIA AND MICROBIAL METABOLITES IN SOYBEAN CROP

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Thesis presented to São Paulo State University, College of Agricultural Sciences, to obtain Doctor of Philosophy degree in Agronomy (Crop Science).

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I dedicate this thesis to my parents Anilson and Suzi, and to my grandfathers Laurindo and Laudelina, and Arnóbio and Maria (*in memory*).

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“Ama e faz o que quiseres.

Se calares, calarás com amor;

se gritares, gritarás com amor;

se corrigires, corrigirás com amor;

se perdoares, perdoarás com amor.

Se tiveres o amor enraizado em ti,

nenhuma coisa senão o amor serão os teus frutos”.

Santo Agostinho

ABSTRACT

The adoption of sustainable production systems has increased considerably in recent years, driven mainly by societal demand for the production of high-quality food under low environmental impact. For soybean (*Glycine max* [L.] Merrill), a broad range of beneficial effects of bacteria have been reported, including biological nitrogen fixation (BNF), plant pathogen biocontrol, plant growth development, and the production of compounds such as lipochitooligosaccharides (LCOs), which activate several physiological and biochemical mechanisms. Rhizobial inoculants and plant growth-promoting bacteria (PGPB) have been widely applied as biofertilizers to legume crops, but plant responses to consortia of other beneficial microbes and microbial molecules have not been comprehensively explored. Therefore, in this study we evaluated the effects of co-inoculating N_2-fixing *Bradyrhizobium japonicum* (strain SEMIA 5079) and *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) with or without foliar application of plant growth-promoting *Azospirillum brasilense* (strains Ab-V5 and Ab-V6) and the biocontrol agent *Bacillus subtilis* (strain QST 713) and seed treatment with microbial secondary metabolites (MSM, rhizobial metabolites enriched in LCOs) on soybean. Experiments were conducted during two growing seasons under greenhouse conditions and three growing seasons under tropical field conditions with a short dry spell at the Lageado Experimental Farm of São Paulo State University (UNESP), Botucatu, São Paulo State, Brazil. We evaluated root system development (directly by optical reading and indirectly by applying rubidium nitrate, $^{85}RbNO_3$), nutritional status, N-ureides, photosynthetic pigments, gas exchange parameters, antioxidant enzyme activities, proline, nodulation, plant growth development, agronomic efficiency index, and soybean grain yield and quality. Combining the beneficial bacterial consortium comprising *Bradyrhizobium* spp. and *A. brasilense* strains with MSM application helped alleviate soybean water stress under field conditions, promoting nodulation, N-ureide content, root and shoot development, photosynthetic activity, the production of photoassimilates and, ultimately, grain filling. Taken together, the results suggest that this combination is an important biotechnological strategy to achieve sustainable food production and security.

Keywords: Antioxidant metabolism. *Azospirillum brasilense*. *Bacillus subtilis*. Biological nitrogen fixation. *Bradyrhizobium diazoefficien*s. *Bradyrhizobium japonicum. Glycine max. Rhizobium tropici.*

RESUMO

A adoção de sistemas de produção mais sustentáveis aumentaram consideravelmente nos últimos anos, impulsionados principalmente pela demanda da sociedade por alimentos com alta qualidade, cuja produção resulta em baixos impactos ambientais. Uma ampla gama de efeitos benéficos por bactérias é relatada na cultura da soja (*Glycine max* [L.] Merrill), incluindo a fixação biológica de nitrogênio (FBN), o biocontrole de patógenos, o desenvolvimento e crescimento, e a produção de compostos como lipo-chitooligossacarídeos (LCOs), ativando vários mecanismos fisiológicos e bioquímicos. Inoculantes rizobianos e bactérias promotoras de crescimento de plantas (BPCP) têm sido amplamente aplicados como biofertilizantes em cultivos de leguminosas, mas as respostas das plantas à consórcios com outros microrganismos benéficos e metabólitos microbianos secundários ainda não são bem explorados. Portanto, neste estudo avaliamos os efeitos da coinoculação por fixadores de N_2 - *Bradyrhizobium japonicum* (estirpe SEMIA 5079) e *Bradyrhizobium diazoefficiens* (estipe SEMIA 5080), com ou sem aplicação foliar do promotor de crescimento de plantas - *Azospirillum brasilense* (estipes Ab-V5 e Ab -V6), e o agente de biocontrole - *Bacillus subtilis* (estirpe QST 713), e com ou sem tratamento de sementes com metabólitos microbianos secundários (MMS, metabólitos rizobianos enriquecidos em lipo-chitooligossacarídeos (LCOs)) na soja durante duas safras em condições de casa de vegetação, e três safras sob condições de campo tropical com curto período de estiagem na Fazenda Experimental Lageado da Universidade Estadual Paulista (UNESP), Botucatu, Estado de São Paulo, Brasil. Neste estudo, avaliamos o desenvolvimento do sistema radicular (por método direto - leitura óptica e método indireto - aplicação de nitrato de rubídio, $^{85}RbNO_3$), estado nutricional, N-ureídeos, pigmentos fotossintéticos, trocas gasosas, atividades de enzimas antioxidantes, teor de prolina, nodulação, desenvolvimento e crescimento das plantas, índice de eficiência agronômica, rendimento e qualidade de grãos de soja. O consórcio bacteriano benéfico compreendendo *Bradyrhizobium* spp. e *A. brasilense* combinado com aplicação de MMS auxiliam no alívio do estresse hídrico da soja em condições de campo, garantindo nodulação, teor de N-ureídeos, desenvolvimento radicular e da parte aérea, melhora a atividade fotossintética da planta e a produção de fotoassimilados e, por fim, o enchimento dos grãos. Tomados em conjunto, os resultados representam uma estratégia biotecnológica importante para alcançar a produção e segurança alimentar sustentável

Palavras-chave: *Azospirillum brasilense*. *Bacillus subtilis*. *Bradyrhizobium diazoefficien*s. *Bradyrhizobium japonicum.* Fixação biológica de nitrogênio. *Glycine max.* Metabolismo antioxidante. *Rhizobium tropici.*

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SUMMARY

GENERAL INTRODUCTION

Symbiosis, like most pathogenic interactions, is characterized by complexity and specificity. Symbiotic processes are of enormous importance for global agricultural productivity and as models for fundamental studies of plant-microorganism interactions (MORETTI et al., 2018). A physiological understanding of the process of biological nitrogen fixation (BNF) and the factors that control it is of paramount importance for both researchers and farmers because such knowledge can support adjustments in crop management to increase nitrogen (N) use efficiency and crop productivity (FUKAMI et al., 2018).

In Brazil, BNF has been successfully exploited through the use of inoculants containing *Bradyrhizobium* spp. to meet the N requirements of soybean cultivation, generating savings for the country of approximately 15 billion dollars annually (HUNGRIA et al., 2015). These diazotrophic bacteria reduce atmospheric nitrogen (N_2) to ammonia (NH_3) through the nitrogenase enzyme complex and proliferate in a wide range of environments with different lifestyles. Depending on their strategies in agricultural systems, diazotrophic bacteria can be classified as symbiotic, associative or free-living (HUNGRIA et al., 2006).

BNF requires abundant energy, and therefore the agronomic significance of free-living N fixers is limited compared with symbiotic fixers. Free-living N fixers colonize plant surfaces and can even invade intercellular spaces but do not form specialized structures and cannot directly transfer NH_3 to the plant (KASCHUK et al., 2010). Thus, it has been suggested that the growth-promoting effects of free-living bacteria could be improved by mechanisms that do not involve BNF (CEREZINI et al., 2016). The entire symbiotic bacterium-plant association process begins with an intense exchange of molecular signals between the organisms involved. The host plant secretes chemotactic substances (sugars, amino acids and carboxylic acids), which stimulate the multiplication of bacteria in the rhizosphere and promote the adherence of rhizobia to the roots of plants (MORETTI et al., 2020).

Many microorganisms synthesize compounds that differ in chemical structure from those found in primary metabolism and that are not essential to the growth and reproduction of the organism (MARKS et al., 2015). This secondary metabolism comprises natural adaptation molecules that are involved in different functions than primary metabolism. The chemical structures of secondary metabolites include βlactams, cyclic peptides, non-protein amino acids, sugars and unusual nucleosides, unsaturated polyacetylenes, nitrile groups, cyclic triesters, terpenoids, and large rings of macrolides (MARKS et al., 2013). Despite not playing key roles in growth and development, microbial secondary metabolites are essential tools for communication and the establishment of rhizobia-legume symbiosis and function as antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, substrates or ligands for enzymes and antagonist and agonist receptors, biocontrol agents, and plant growth-promoting molecules (GOUGH; JACQUET, 2013).

We hypothesized that beneficial microbes and their metabolites increase soil exploration by the root system, photosynthetic activity, and plant nutrition and development and alleviate water stress in soybean by increasing the intrinsic tolerance of soybean plants to abiotic stresses, thereby improving yield. To test this hypothesis, we evaluated root system activity and development, photosynthetic pigments, gas exchange parameters, antioxidant enzyme activities, proline content, nodulation, plant growth development and grain yield in soybean inoculated with beneficial bacterial consortia (N_2-fixing *Bradyrhizobium* spp. with different combinations of *Azospirillum brasilense* strains, *Bacillus subtilis*, and rhizobial metabolites) during two growing seasons under greenhouse conditions and three growing seasons under tropical field conditions.

GENERAL CONCLUSIONS

The beneficial bacterial consortium comprising seed inoculation with N_2-fixing *Bradyrhizobium japonicum* (strain SEMIA 5079) and *Bradyrhizobium diazoefficiens* (strain SEMIA 5080) combined with seed treatment with MSM (rhizobial metabolites enriched in LCOs) and foliar spraying in the V_4 soybean growth stage with plant growth-promoting *Azospirillum brasilense* (strains Ab-V5 and Ab-V6) increased root system soil exploration and, in turn, the distribution and activity of soybean roots.

This beneficial bacterial consortium also increased nodulation, the production of N-ureides and, consequently, N nutrition. The combination of bacterial consortia and metabolites may represent an effective strategy to mitigate dry spell stress during soybean development by reducing the leaf contents of hydrogen peroxide and proline, lipid peroxidation, and the activities of the enzymes superoxide dismutase, catalase, and ascorbate peroxidase, resulting in greater soybean photosynthetic potential.
Ultimately, the combination of this bacterial consortium and MSM increased both grain yield (~11%) and crude protein concentration. This study illustrates the feasibility of identifying beneficial bacterial consortia that can promote plant growth and elicit plant tolerance to abiotic stress, which represents an important biotechnological strategy to achieve sustainable food production and security.

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