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VANESSA FOGAÇA DE FREITAS DUIN

ESTRATÉGIAS DE MITIGAÇÃO DOS EFEITOS DA RESTRIÇÃO HÍDRICA EM SOJA

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina - UEL, como requisito para a obtenção do título de Doutora em Microbiologia.

Orientador: Dr. Marco Antonio Nogueira

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DUIN, Vanessa Fogaça de Freitas. **Estratégias de mitigação dos efeitos da restrição hídrica em soja**. 2022. 88 f. Tese (Doutorado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2022.

RESUMO

A soja [*Glycine max* (L.) Merril] é a principal cultura do agronegócio brasileiro e o país se destaca pelo uso da fixação biológica de nitrogênio (FBN) com estirpes de *Bradyrhizobium* spp. para o suprimento de N à cultura. No entanto, a seca prejudica o processo de FBN e, consequentemente, o desenvolvimento e rendimento de grãos. Esse trabalho objetivou avaliar estratégias que reduzam o efeito da seca na soja pelo aumento da eficiência da FBN, seja por meio da interação entre genótipos de soja tolerantes à seca e estirpes de *Bradyrhizobium*, seja pela reaplicação de micronutrientes essenciais à FBN e reinoculação da soja após um período de seca. Em condições hidropônicas em casa de vegetação, genótipos de soja melhorados para a tolerância à seca foram inoculados com estirpes de *Bradyrhizobium*, simulando-se exposição à seca pelo uso de polietilenoglicol 6000. As respostas fisiológicas, bioquímicas e moleculares das plantas foram avaliadas para melhor compreensão dos efeitos da seca e identificar a interação planta-estirpe mais promissora para tolerância à escassez hídrica. A inoculação da estirpe CNPSo 1448 no genótipo BRS 284 acarretou diminuição da abertura estomática, maior acúmulo de prolina nos nódulos e redução na atividade da superóxido dismutase (SOD) e maior expressão de genes envolvidos na proteção celular e enzimas do estresse oxidativo. Para PI 471938, houve economia de água pela diminuição da abertura estomática e redução da transpiração, para BR14-4760 houve aumento do teor de prolina, redução da atividade da catalase (CAT) na folha e redução da SOD nos nódulos. A estirpe CNPSo 06 favoreceu um melhor desempenho na interação com os genótipos sob seca apresentando aumento do teor de prolina para BRS 284 e PI 471938, além do aumento da atividade enzimática no nódulo da PI 471938. Para BR14-4760 houve aumento na taxa fotossintética e redução na temperatura foliar, com diminuição da CAT foliar e indução da expressão para todos os genes avaliados nos nódulos. Assim, a combinação BR14-4760 com a estirpe CNPSo 06 foi a mais eficaz na indução de respostas de defesa da planta ao déficit hídrico. A campo, foram testados os efeitos da reaplicação de cobalto e molibdênio via foliar e reinoculação de *Bradyrhizobium*, *Azospirillum brasilense* e coinoculação de ambos na cultura de soja após período de seca. O genótipo DM 6563 IPRO apresentou efeito negativo após reaplicação de CoMo no período vegetativo sob estiagem e a reinoculação não favoreceu uma melhor resposta após período de seca. Para o genótipo BMX PONTA IPRO, que sofreu um menor período de seca, a reaplicação de CoMo favoreceu o aumento na produtividade, número de vagens e grãos, peso total de grãos e peso de cem grãos. A reaplicação desses micronutrientes via foliar na fase inicial do estádio reprodutivo na cultura da soja pode favorecer um melhor desempenho da cultura após período de seca. Portanto, a integração de estratégias de mitigação para alívio da seca é uma ação promissora na manutenção da fixação biológica de nitrogênio para o melhor enfrentamento da soja ao estresse hídrico.

Palavras-chave: estresse hídrico; inoculação; metabolismo de N; RPCP; fisiologia vegetal.

DUIN, Vanessa Fogaça de Freitas. **Strategies to mitigate the effects of water restriction on soybean**. 2022. 88 p. Thesis (Ph.D. in Microbiology) – State University of Londrina, Londrina, 2022.

ABSTRACT

Soybean [*Glycine max* (L.) Merril] is the main crop of Brazilian agribusiness and the country stands out for the use of Biological Nitrogen Fixation (BNF) with strains of *Bradyrhizobium* spp. to supply N to the crop. However, drought impairs the BNF process and, consequently, the development and yield of grains. This study aimed to evaluate strategies that reduce the effect of drought on soybeans by increasing the efficiency of BNF, either through the interaction between drought tolerant soybean genotypes and *Bradyrhizobium* strains, or through the reapplication of essential micronutrients to BNF and soybean re-inoculation after a period of drought. Under hydroponic conditions in a greenhouse, in which soybean genotypes improved for drought tolerance were inoculated with *Bradyrhizobium* strains, the exposure to drought was simulated by using polyethylene glycol 6000. The physiological, biochemical, and molecular responses of plants were evaluated to better understand the effects of drought and identify the most promising plant-strain interaction for water scarcity tolerance. The inoculation of the CNPSo 1448 strain in the BRS 284 genotype caused a decrease in the stomatal opening, greater accumulation of proline in the nodules, a reduction in superoxide dismutase (SOD) activity and greater expression of genes involved in cell protection and enzymes from oxidative stress. For PI 471938, there was water savings due to the reduction of stomatal opening and reduction of transpiration, for BR14-4760 there was an increase in the proline content, reduction of catalase activity (CAT) in the leaf and reduction of SOD in the nodules. The CNPSo 06 strain favored a better performance in the interaction with the genotypes under drought, showing an increase in the proline content for BRS 284 and PI 471938, in addition to an increase in enzymatic activity in the nodule of PI 471938. For BR14 4760, there was an increase in photosynthetic rate and a reduction in leaf temperature, with a decrease in leaf CAT and induction of expression for all genes evaluated in the nodules. Thus, the combination BR14-4760 with the CNPSo 06 strain was the most effective in inducing plant defense responses to water deficit. In the field, the effects of reapplication of Cobalt and Molybdenum via foliar and re-inoculation of *Bradyrhizobium*, *Azospirillum brasilense* and co-inoculation of both in the soybean crop after a dry period were tested. The DM 6563 IPRO genotype showed a negative effect after reapplication of CoMo in the vegetative period under drought and the re-inoculation did not favor a better response after the drought period. For the BMX PONTA IPRO genotype, which suffered a shorter period of drought, the reapplication of CoMo favored an increase in productivity, number of pods and grains, total grain weight and one hundred grain weight. The reapplication of these micronutrients via foliar in the initial phase of the reproductive stage in the soybean crop may favor a better performance of the crop after the dry period. Therefore, the integration of mitigation strategies for drought relief is a promising action in the maintenance of FBN for better coping of soybeans with water stress.

Keywords: water stress; inoculation; N metabolismo; PGPR; plant physiology.

SUMÁRIO

INTRODUÇÃO

A soja [*Glycine max* (L.) Merril] é uma leguminosa com alto teor proteico e apresenta grande relevância na economia mundial devido à ampla gama de usos, sendo utilizada na alimentação humana, animal, como biocombustível, além de diversas aplicações industriais. O Brasil atualmente é o maior produtor mundial do grão, com a produção total de 123,83 milhões de toneladas (CONAB, 2022), e o uso de inoculantes com estirpes elites de *Bradyrhizobium* no cultivo é um dos principais fatores que vêm contribuindo para o destaque na sua produtividade e sustentabilidade.

Essa associação benéfica entre leguminosas e rizobactérias leva à formação de estruturas especializadas nas raízes denominadas nódulos, em cujo interior há suprimento de fontes de carbono à bactéria pela planta e, em contrapartida, a bactéria disponibiliza nitrogênio (N) na sua forma assimilável para a planta. Assim, a fixação biológica de N (FBN) na soja fornece quase todo o N necessário para o desenvolvimento e alto rendimento da cultura, dispensando em grande parte dos casos a necessidade da adubação com N mineral.

No entanto, apesar dos avanços no sistema de cultivo, as intempéries climáticas ainda são fatores que podem causar danos nas lavouras, com grandes perdas econômicas, sendo a seca o fator abiótico mais limitante. Por isso, nos últimos anos o uso de novas tecnologias como genômica, transcriptômica, e transformação genética tem sido ferramentas que auxiliam para uma melhor compreensão dos mecanismos de proteção das plantas contra o estresse abiótico, contribuindo com os programas de melhoramento genético no desenvolvimento de genótipos mais tolerantes à seca (Lamaoui et al., 2018; Aleem et al., 2020; Molinari et al., 2020).

A seleção de genótipos com FBN tolerante à seca tem sido um fator empregado na seleção de genótipos tolerantes à seca em programas de melhoramento da soja devido a importância da manutenção de uma FBN eficiente sob condições de estresse, uma vez que, este processo necessita de uma hidratação adequada, seja para o transporte dos produtos da fixação, seja para o suprimento de fontes de energia para os bacteroides. O genótipo PI 471938 vem sendo utilizado como fonte de variabilidade genética para introdução da característica de FBN tolerante à seca por expressar a característica de murcha lenta (slow-wilting) em estudos de campo (King, Purcell e

Brye, 2009) e manutenção da FBN sob condições de secagem do solo (Devi e Sinclair, 2013)

Além da variação genotípica entre genótipos de soja quanto à capacidade de manter o processo simbiótico em condições de restrição hídrica, há também diferenciação na resposta quanto ao simbionte envolvido. O genótipo DT2008 apresentou maior crescimento e melhor desenvolvimento de nódulos comparada ao genótipo Williams 82 (W82), sob condições suficientes de água, déficit hídrico e reidratação posterior (Sulieman et al., 2015). Assim, é importante considerar a interação entre genótipos tolerantes e estirpes de *Bradyrhizobium* na seleção de genótipos de soja para um melhor desempenho sob restrição hídrica.

Outras estratégias também podem ser exploradas para a melhoria do processo de FBN frente ao estresse hídrico. A combinação de rizobactérias promotoras do crescimento de plantas (RPCP) no cultivo tem aumentado a eficiência da FBN, sendo a coinoculação com *Azospirillum* um grande aliado na última década para o aumento da produtividade da soja (Cerezini et al., 2016; Fukami et al., 2018; Rondina et al., 2020). A adição de metabólitos secundários microbianos (MSM) em inoculantes também aumentou a tolerância da leguminosa contra a seca, uma vez que, estimulam a nodulação precoce, e promovem aumento de raízes laterais que beneficiam a cultura (Cerezini et al., 2016 e Gomes et al., 2019).

A aplicação dos micronutrientes Cobalto (Co) e Molibdênio (Mo) também tem beneficiado o plantio, pois, são cofatores enzimáticos do processo de FBN que podem aumentar a eficácia da fixação permitindo um maior acúmulo de N nas fases iniciais para posterior translocação destes na formação de grãos (Campo et al., 2009). Com isso, o suprimento adequado de Co e Mo podem reduzir os impactos negativos da seca na produtividade sendo mais uma possível alternativa para lidar com a seca. Assim, os avanços em pesquisas e a aplicação de diferentes estratégias como medida para reduzir os impactos da seca na cultura da soja, tem sido crescente. No entanto, se essas estratégias forem trabalhadas em conjunto poderá gerar resultados mais efetivos no combate aos danos causados à cultura, manutenção da FBN, e melhor rendimento do grão.

O objetivo deste trabalho foi (1) avaliar a interação entre genótipos de soja, com FBN sensível e tolerante a seca, com diferentes estirpes de *Bradyrhizobium* sob simulação de estresse hídrico com Polietilenoglicol (PEG 6000) em casa de vegetação; (2) avaliar o efeito da reaplicação de CoMo e a reinoculação de *Bradyrhizobium*, *A. brasilense* e coinoculação de ambos na FBN e produtividade da cultura da soja após período de seca em condições de campo.

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CAPÍTULO 1

(REVISÃO DE LITERATURA)

ESTRATÉGIAS PARA LIDAR COM O ESTRESSE HÍDRICO NA FIXAÇÃO BIOLÓGICA DE NITROGÊNIO EM SOJA

ESTRATÉGIAS PARA LIDAR COM O ESTRESSE HÍDRICO NA FIXAÇÃO BIOLÓGICA DE NITROGÊNIO EM SOJA

RESUMO

O sistema de produção da soja [*Glycine max* (L.) Merrill] no Brasil e em outros países da América do Sul conta com a fixação biológica de nitrogênio (FBN) que, em simbiose com estirpes elite de *Bradyrhizobium*, supre praticamente toda a necessidade de N da cultura, cerca de 83 kg por tonelada de grãos. No entanto, a seca prejudica vários processos metabólicos das plantas, especialmente a FBN, limitando o desenvolvimento e a produtividade. Estratégias para lidar com a seca compreendem o melhoramento genético da planta hospedeira, a seleção de linhagens de rizóbios para maior tolerância ao estresse hídrico, coinoculação com outros microrganismos promotores de crescimento de plantas e seus metabólitos para estimular a simbiose. Um melhor entendimento dos mecanismos fisiológicos envolvidos na simbiose relacionada à planta hospedeira e ao simbionte são cruciais para o desenvolvimento de inoculantes, e de genótipos de soja mais adaptados à seca, garantindo menor comprometimento da seca sobre o fornecimento de N à cultura. O manejo do solo baseado no sistema plantio direto e a melhoria de suas características químicas e biológicas para permitir que as raízes alcancem camadas mais profundas também são estratégias aliadas para lidar com a seca. A manutenção da cobertura morta sobre a superfície do solo e o aumento dos teores de matéria orgânica, além da melhoria das condições químicas, permite maior acesso à água no perfil do solo, e uma melhor condição para a simbiose e outros processos biológicos benéficos. Estratégias para estimular ou manter a FBN podem ser uma das principais ações para mitigar o estresse hídrico em soja. Esta revisão discute o progresso obtido até o momento em estratégias que visam diminuir o comprometimento da FBN sob seca e sugere o uso de ferramentas integradas para aliviar os efeitos da seca na cultura da soja para uma produtividade mais estável e sustentável.

Palavras-chave: *Azospirillum*, co-inoculação, tolerância à seca, N-metabolismo, murcha-lenta, manejo do solo.

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Review

Strategies to deal with drought-stress in biological nitrogen fixation in soybean

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ABSTRACT

The soybean [*Glycine* max (L.) Merrill] production system in Brazil and other South American countries relies on the biological nitrogen fixation (BNF) in symbiosis with *Bradyrhizobium* elite strains, which provides nearly all the crop N requirements, about 83 kg per ton of grains. However, drought impairs several plant metabolic processes, especially BNF, limiting development and yield. Strategies to deal with drought comprise breeding, including genetic modification of the host plant, selection of rhizobial strains for more tolerance to water stress, co-inoculation with other plant growth-promoting microorganisms, and their metabolites to stimulate the symbiosis. A better understanding of physiological mechanisms involved in the symbiosis related to the host plant and the symbiont are crucial to development inoculants, and also soybean genotypes more adapted to drought, assuring lower impairment of drought on the N supply to the crop. Soil management based on no-till, and chemical and biological improvement to allow roots to reach deeper layers are also allied strategies to deal with drought. The maintenance of mulching and increase of soil organic matter levels, in addition to improvement of soil chemical conditions, allow more access to water in the soil profile, and a better condition for the symbiosis and other beneficial biological processes. Strategies to stimulate or maintain BNF can be one of the main strategies to mitigate drought stress in soybean. This review discusses the progress so far on strategies aiming to lessen the impairment of BNF under drought and suggests using integrated tools to alleviate the effects of drought on the soybean crop for a more stable and sustainable yield.

1. Introduction

The soybean crop [*Glycine* max (L.) Merril] is among the most relevant oilseed worldwide (Herridge et al., 2008), which main producers and exporters are Brazil, the United States, and Argentina (USDA, 2021). Soybean demands 83 kg of nitrogen (N) per ton of grains, and there is a positive correlation between total assimilated N during the vegetative and beginning of reproductive stages with grain yield (Embrapa, 2020). In South America, the N acquisition by soybean is mostly based on the biological nitrogen fixation (BNF) process upon inoculation with elite strains of *Bradyrhizobium*, and dismisses the need for supplemental mineral N fertilizers. In Brazil, the non-use of chemical N fertilizer in soybean results in an economy estimated at US$ 15 billion annually, in addition to environmental advantages (Hungria and Mendes, 2015). However, despite technological advances in the production system, the occasional unevenness of rainfall during the crop season may impair the BNF process and limit the crop yield, given that the majority of the cropping system is non-irrigated.

In recent years, occurrences of extreme weather events such as drought, heavy rainfall, heat waves, and tropical cyclones, have threatened crop yields and food security (IPCC, 2014). Rainfall reduction by up to 20% is predicted in subtropical areas by 2100 (IPCC, 2007), and more frequent dry periods have been observed during the wet season, causing losses to the agribusiness chain (Assad et al., 2013; Zilli et al., 2020).

Drought decreased soybean yield by 11.8% in Brazil during the 2011/2012 crop season, which represented less 8.9 million tons of grains compared with the previous season (CONAB, 2012). In the United States, drought caused severe losses in the 2015/16 crop season; in Argentina, drought reduced soybean yield by more than 30% in the 2017/18 crop season (USDA, 2018). In the 2019/20 crop season, the United States yield dropped due to delay in the sowing caused by

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drought. In Argentina, drought and high temperatures reduced the yield potential by 8% in 2019/20 compared with the previous season (USDA, 2021). These statistics highlight the seasonality of soybean yield caused by drought, leading to economic losses and food insecurity.

Crop response to drought is considered a syndrome, i.e., a pool of simultaneous responses (Lawlor, 2002). Water deficit impairs all stages of soybean cycle, from germination up to formation and filling of grains, as well as its quality in terms of protein and oil contents (Manavalan et al., 2009). Soybean water requirement increases with development, reaching 7 to 8 mm day^{-1} at flowering/filling of grains, and decreasing thereafter (Embrapa, 2020).

The BNF process is especially vulnerable to drought, and drops even before the transpiration and photosynthetic rates (King and Purcell, 2005; Arrese-Igor et al., 2011; Bellaloui and Mengistu, 2015). Several mechanisms involved in the limitation of the BNF under drought are related to the host plant (Gálvez et al., 2005; Cerezini et al., 2014; Sinclair and Nogueira, 2018; Nogueira et al., 2020). Concerning the bacteria, *Bradyrhizobium* strains have different levels of tolerance to drought and more tolerant ones may help the host plant to deal with water restriction (Kunert et al., 2016; Cerezini et al., 2020). Thus, the maintenance of the BNF process under drought is essential for helping the crop to deal with drought stress in BNF-relying soybean production systems.

Negative impacts of climate changes on agricultural production reinforces the need for more resilient and environmentally sustainable practices in the agricultural areas not only in Brazil (Zilli et al., 2020), but also in a global scale. For soybean in particular, integrated practices like plant breeding for drought tolerance, selection of more adapted rhizobial strains, drought stress-alleviating plant growth-promoting microorganisms and their metabolites, plant nutrition, and soil management, would contribute to relief stress caused by drought and make yields more stable over unfavorable climate, as will be further commented in this review.

This review highlights the negative effect of drought on the BNF in soybean, and discusses strategies to reduce the impairment on the crop N supply and yield based on the host plant, the symbiont, plant growth-promoting microorganisms and their secondary metabolites, and soil management. Discussions on research gaps on drought tolerance and actions to fill them are also presented.

2. The BNF process in soybean

Along with water, N is the most limiting factor for plant growth and development. Its role in structural, genetic, and metabolic functions is pivotal for crop yields (Christophe et al., 2011). Although N_2 is the most abundant constituent of the Earth's atmosphere, none eukaryotic organism is able to use it. The triple covalent bonds between the N atoms make N_2 highly stable and non-reactive (Hoffman et al., 2014). However, as a result of million years of evolution, some species belonging to the Leguminosae (=Fabaceae) family have developed the ability to establish symbiosis with diazotrophic bacteria of the α-Proteobacteria and β-Proteobacteria classes, collectively named rhizobia, which are capable to make N available to plants by means of the BNF (Baral et al., 2016; Sprent et al., 2017). This is a low-cost N source, sufficient to keep high soybean yields, with low environmental impact, and dismisses the need for mineral N fertilizer (Hungria and Mendes, 2015; Kaschuk et al., 2016).

The symbiosis involves several steps that begin with the attraction of the bacteria by molecular signals, usually flavonoids, excreted by the host plant in the rhizosphere. In the infection and colonization process, physiological, biochemical, and morphological changes occur in the host plant and in the microorganism (Baral et al., 2016) that result in the nodule formation (Ribeiro et al., 2014; Keller and Lau, 2018). Within the nodule, the bacteria are differentiated into bacteroids and several proteins are synthesized, such as the nitrogenase complex and the leghemoglobin, starting the BNF process (Hoffman et al., 2014; Ribeiro et al., 2014).

The nitrogenase complex consists of two units, iron-protein (Fe-protein) and molybdenum-iron-protein (MoFe-protein) that, along with ferridoxin, carry electrons for reduction of N_2 to ammonia (NH_3), which is rapidly converted into ammonium (NH_4^+) and released by diffusion into the cytosol of the infected cells. As the accumulation of NH_4^+ is toxic to the bacteroid and to the host cell, it must be converted into organic forms, such as aminoacids, amides, or ureides, like in soybean, for translocation and later incorporation into the plant N metabolism (Sprent, 1980; Hoffman et al., 2014; Baral et al., 2016).

In general, legumes of temperate regions transport the fixed N as amides, but soybean transports about 90% of its fixed N as ureides (King and Purcell, 2005; Sinclair et al., 2007; Christophe et al., 2011). The main ureides are allantoin, synthesized in the peroxisomes from uric acid, and allantoic acid, synthesized in the endoplasmic reticulum, from allantoin (King and Purcell, 2005). These N compounds are then translocated, via xylem, from nodules to shoots, where they are rapidly assimilated (King and Purcell, 2005; Alamillo et al., 2010). Assimilation reactions are usually mediated by the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT), which activities can be indicators of N assimilation efficiency, and indirectly, the BNF activity (Hungria et al., 1991; Christophe et al., 2011; Dunn, 2015; Cerezini et al., 2020). Selection of plant genotypes able to keep the GS activity under drought could be one more promising trait for keeping the BNF process under drought (Cerezini et al., 2019).

Leghemoglobin regulates the transport of O_2 to the N_2-fixing cells in nodules, as free O_2 irreversibly inactivates the nitrogenase, so that O_2 concentration is maintained at a non-deleterious level, but still allows the respiration of bacteroids (Sinclair and Nogueira, 2018). Active nodules show intense internal pink color, indicating the leghemoglobin activity (Sprent, 1980). Cobalt (Co) is involved in the synthesis of leghemoglobin and thus it is also important for the BNF process. Thus, nutrients directly involved in the BNF like Co must be considered as strategy to improve the BNF efficiency to help the plant to deal with drought stress.

BNF is a high energy-demanding process from the host plant to provide energy to the microsymbiont and C skeletons to the N assimilation processes (Belane and Dakora, 2015; Mapope and Dakora, 2016). The energy invested in N acquisition via BNF is higher than its uptake from soil as nitrate (NO_3^-), but the sink of photoassimilates caused by the symbiosis stimulates an increase up to 28% in the photosynthetic rate, compensating losses (Kaschuk et al., 2009). As N is involved in the synthesis of chlorophylls and Rubisco, BNF also increases the photosynthetic efficiency by supplying N to the host plant (Kaschuk et al., 2010; Belane and Dakora, 2015; Mapope and Dakora, 2016; Cerezini et al., 2020). The stimulation of photosynthesis due to BNF also delays the degradation of leaf proteins and chlorophyll, and thus decreases the plant senescence rates that results in more and heavier grains, and consequently, higher yields (Kaschuk et al., 2010). These statements suggest that the maintenance of BNF and stimulation of photosynthesis can contribute to relief the negative effects of drought. On the other hand, the host plant needs to close stomata to save water, and thus reduce the photosynthetic rates, limiting the BNF due to shortage of energy sources to the bacteroids. This paradox can be solved by using integrated strategies, in which the symbiont, the host plant, and the environment should be managed toward a resilient system to face water restriction as will be later discussed.

3. Drought effects on BNF in soybean

Drought is considered the main restrictive environmental factor to BNF, with negative consequences on yield (Ladrera et al., 2007; Sinclair et al., 2007; Cerezini et al., 2016a). The BNF process in soybean is particularly vulnerable to drought, being impaired even before the transpiration rate and photosynthesis (King and Purcell, 2005; Arrese-Igor et al., 2011; Bellaloui and Mengistu, 2015; Cerezini et al., 2020).

Water restriction reduces the rhizobial survival in the soil (Zilli et al., 2013) and the establishment of the symbiosis, leading to reduction in the number, mass and metabolism of nodules, in addition to reduction in the nitrogenase activity (Christophe et al., 2011; Prudent et al., 2015). The initial steps of the symbiosis establishment, especially exchanges of molecular signals between the host plant and rhizobia, can be impaired by drought. Given that the exchange of signals depends on diffusion of such molecules in the soil solution, water restriction can impair this important early step of the symbiosis. Thus, management strategies to conserve water in the soil will also contribute for the early steps of the establishment of the symbiosis.

Once the symbiosis have established and nodules have been formed, three main mechanisms related to the host plant are suggested as responsible for reduction/inhibition of BNF under drought: (1) oxygen limitation inside the nodules; (2) shortage of carbon substrates as energy source to the bacteroids; (3) feedback by N-compounds as ureides (Gálvez et al., 2005; Ladrera et al., 2007; Alamillo et al., 2010; Cerezini et al., 2014; Cerezini et al., 2020). Thus, the optimization of these mechanisms would be reached not only by plant breeding (Sinclair and Nogueira, 2018), but also the integration with soil management for water conservation and plant nutrition. For example, some metabolic ways for metabolism of ureids are Mn-dependent. Thus, supplemental Mn fertilization would optimize the metabolism of ureids and reduce the probability of negative feedback caused by non-metabolized ureids (Purcell et al., 2000; Vadez et al., 2000).

The nodule permeability to gases decreases under water restriction and limits the respiration of the bacteroid because the O_2 flow through the dehydrated cortical cells of the nodules is reduced (Hungria and Vargas, 2000; Christophe et al., 2011; Sinclair and Nogueira, 2018). Moreover, the content of leghemoglobin decreases under severe drought (Manavalan et al., 2009), compromising the supply of O_2 to the microsymbiont; as a consequence, the nitrogenase activity decreases (Christophe et al., 2011). Water availability is also essential for transportation and assimilation of photoassimilates in nodules, as well as to exportation and metabolism of N-compounds (notably ureides) in the shoots. The impairment of these processes caused by water restriction disrupts the C and N metabolism that regulates the BNF in nodules (Ladrera et al., 2007; Alamillo et al., 2010; Arrese-Igor et al., 2011; Cerezini et al., 2014; Prudent et al., 2015; Cerezini et al., 2017; Cerezini et al., 2020).

Sucrose synthase (SS) is the main enzyme involved in the hydrolysis of sucrose, but reduction in SS activity under drought leads to sucrose accumulation in nodules because bacteroids cannot use it as energy source (González et al., 1995), leading to starvation of the microsymbiont (Gálvez et al., 2005). The lack of dicarboxylic acids, mainly malate and succinate, used as energy and reducing power, decreases the nitrogenase activity and the BNF process (Gálvez et al., 2005; Ladrera et al., 2007; Arrese-Igor et al., 2011). In fact, SS activity in soybean genotypes showing N-fixation tolerant to drought remained higher than in susceptible genotypes (Ladrera et al., 2007), suggesting that plant selection for this trait could help to increase soybean tolerance to drought by improving the BNF.

Ureides are the main BNF compounds exported from nodules to shoots in soybean (Sprent, 1980; Alamillo et al., 2010; Baral et al., 2016). The advantage of ureides in the transport of the fixed N is the lower ATP cost for synthesis: 1–4 ATP, compared with 5–7 ATP spent in the production of glutamate and asparagine (Minchin et al., 1981). However, ureides have low solubility, which implies the need of 2.6 times more water for transporting equivalent amounts of N as asparagine, for example (Sprent, 1980). Consequently, there is an inverse relationship between water availability in the soil and transport of ureides to shoots. A positive correlation between BNF sensitivity to drought and accumulation of ureides in different parts of soybean plants like leaf blade, petioles, sap or nodules have been observed (Devi and Sinclair, 2013; Cerezini et al., 2014; Marquez-Garcia et al., 2015; Cerezini et al., 2017; Cerezini et al., 2020). Soybean varieties susceptible to drought have more accumulation of ureides in shoots (King and Purcell, 2005; Devi and Sinclair, 2013; Cerezini et al., 2014; Cerezini et al., 2017; Cerezini et al., 2020) due to decreased metabolism of ureides in leaves, which may accelerate leaf senescence (King and Purcell, 2005). In addition, accumulation of ureides in nodules is toxic to the bacteroids and negatively affects the nitrogenase activity in a feedback mechanism (Hungria and Vargas, 2000; Alamillo et al., 2010; Cerezini et al., 2014; Cerezini et al., 2017; Cerezini et al., 2020). Plants that transport N-compounds as amides are less sensitive to drought than plants that transport N as ureides (Sinclair et al., 2007). A summary of the main negative effects of drought on soybean BNF is represented in Fig. 1.

4. Strategies to increase BNF drought tolerance in soybean

As part of the evolutionary process, plants have developed mechanisms to deal with drought. A number of defense mechanisms can be activated, mediated by several genes coding for proteins that act synergistically in metabolic pathways aiming at increasing tolerance/resistance to water restriction (Shinozaki and Yamaguchi-Shinozaki, 2007).

Stomatal closure under water restriction leads to negative consequences on gas exchanges, impairing transpiration, photosynthesis, growth, reproduction, enzymatic activities, metabolism of N and C, among other metabolic processes (Sinclair et al., 2007; Christophe et al., 2011; Cerezini et al., 2017; Cerezini et al., 2020), with negative effect on yield. However, morphophysiological stress-induced mechanisms of acclimatization may reduce the loss of water (Cattivelli et al., 2008; He et al., 2017), like partial stomatal closure and reduction of transpiration rates (Flexas et al., 2006); limitation of shoot growth to save energy and elongation of the root system to search for available water in the soil (Cattivelli et al., 2008; Sulieman et al., 2015; Cerezini et al., 2019); savings in daily water use before flowering to be used thereafter, ensuring reproduction (He et al., 2017); osmotic adjustment by accumulation of solutes that allow reduction of the osmotic potential and maintenance of the cell turgor pressure (Cattivelli et al., 2008; Cerezini et al., 2017). In addition, plants may accelerate senescence and abscission of older leaves, a process also known as leaf area adjustment (Gepstein and Glick, 2013). Soybean plants may induce the senescence of older leaves, which have lower photosynthetic capacity, and thus assure more organic compounds to the nodules for maintenance of the BNF and decreased leaf area to reduce water loss (Marquez-Garcia et al., 2015). However, leaf senescence can decrease the yield potential, but this is still advantageous facing the risks of greater losses if nodules starved and no available water were saved in the soil for a further use. Thus, we must take in consideration that tolerance to drought not necessarily implies in suppressing losses, but relieving the losses to the lowest possible level.

New technologies such as genomics, transcriptomics, and genetic transformation have enabled better understanding of the mechanisms of plant protection against abiotic stresses and have enabled the development of stress-tolerant crops (Lamaoui et al., 2018; Aleem et al., 2020). The understanding of gene expression induced by water restriction in soybean can also be a useful tool to clarify the involved mechanisms (Fuganti-Pagliarini et al., 2017; Molinari et al., 2020) and seek for strategies to alleviate the stress. Although molecular studies involving gene expression in leaves and root tissues of the host plant have advanced in recent years, there is still a need for studies on gene expression within the nodule environment, and in this case, not only genes related to the host plant, but also to the symbiont bacteria, to better understand the mechanisms the symbiosis responds to drought. Thus, selection of traits related to tolerance to drought could be assisted by molecular techniques under artificial conditions in the laboratory or in the greenhouse, saving efforts and time in programs aiming to improve soybean tolerance to drought.

Specific cell receptors recognize the stressing condition and induce a cascade of events that lead to the expression of several gene categories (Shinozaki and Yamaguchi-Shinozaki, 2007). Some dehydration

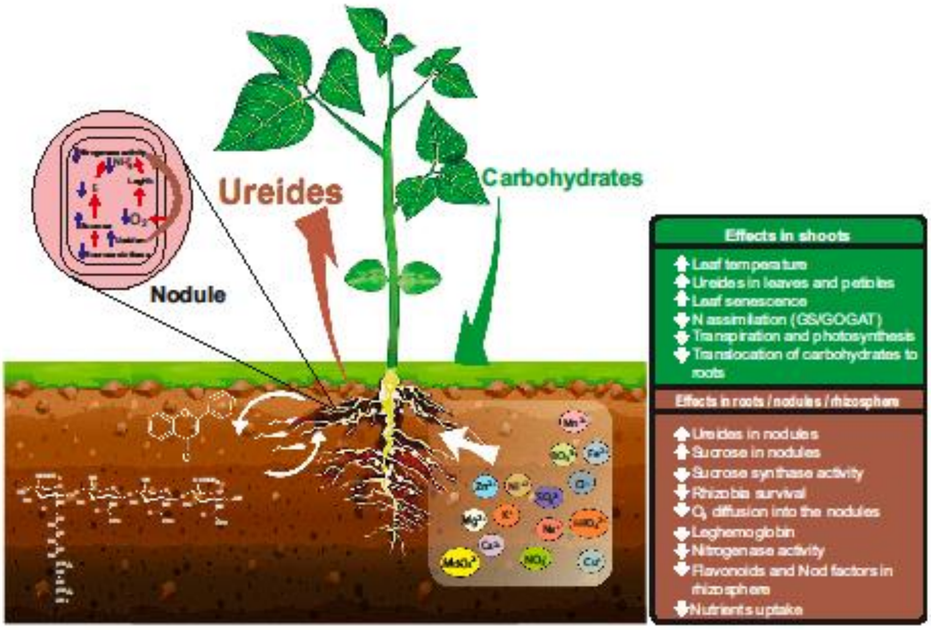

Fig. 1. Summarization of the main effects of drought on the impairment of the biological nitrogen fixation (BNF) process in soybean. Drought may affect the establishment of the symbiosis by impairing the signaling between plants and bacteria. Once established, drought may impair the transportation of ureides from nodules to shoots, and the flow of carbohydrates from shoots to nodules. The green box summarizes the processes that are impaired in the shoots; the brown box summarizes the processes that are impaired in the soil. Arrows up indicate increase of the trait or process; arrows down indicate reduction of the trait or process. Red arrows within the nodule indicate the flow

responsive genes belonging to the family of genes that encode transcription factors called DREB ("Dehydration Responsive Element Binding Protein") have been identified (Yamaguchi-Shinozaki and Shinozaki, 2005). These transcription factors trigger the activation of other genes that lead to the protection of cell structures during dehydration (Yamaguchi-Shinozaki and Shinozaki, 2005). The genetic introduction of DREB transcription factor has been made in plants such as tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), wheat (*Triticum* spp.), soybean, and cotton (*Gossypium hirsutum*) to induce drought tolerance (Yamaguchi-Shinozaki and Shinozaki, 2005; Chen et al., 2007b), as well as other families of transcription factors involved with drought tolerance like WRKY, ZIP, ZFP, MYB, NAC, and ERFs (Fuganti-Pagliarini et al., 2017; Zhao et al., 2018; Aleem et al., 2020). This strategy can cut edges in the process of obtaining genotypes resistant to drought, but the need for deregulation of genetically-modified organisms in several countries may become this process costly economically.

4.1. *Plant genotypic variation*

Plants in general have intrinsic mechanisms to deal with abiotic stresses, which may vary among genotypes within a given species. The characterization and identification of traits in tolerant genotypes can represent a strategy to increase yield stability under drought (Devi et al., 2014; Sinclair and Nogueira, 2018; Nogueira et al., 2020), by means of identification of genotypes containing traits to be used for crossings in breeding programs aiming at tolerance to drought (Purcell et al., 2000).

Genotypic variations among soybean genotypes for BNF sensitivity to drought have been largely documented (Serraj and Sinclair, 1997; Sinclair et al., 2007; Arrese-Igor et al., 2011; Cerezini et al., 2014; Devi et al., 2014; Fenta et al., 2014; Sulieman et al., 2015; Engels et al., 2017; Zhao et al., 2018; Cerezini et al., 2020). For example, the 'Jackson' cultivar was selected in 1987 among 28 genotypes as having BNF tolerant to drought (Sall and Sinclair, 1991) and later this trait was confirmed among 18 soybean cultivars (Serraj and Sinclair, 1997). The physiological advantage of 'Jackson' was attributed to its ability to rapidly metabolize ureids, an advantage under drought as discussed above. This capacity was associated with higher Mn accumulation in shoots (Serraj and Sinclair, 1997; Purcell et al., 2000; Cerezini et al., 2017), since the enzyme amidohydrolase involved in the metabolism of ureides requires Mn as cofactor (Vadez et al., 2000). However, there are other Mn-independent pathways involved in the metabolism of ureides (Charlson et al., 2009; Alamillo et al., 2010), so that the search for Mn-accumulating genotypes not necessarily will result in higher capacity to metabolize ureides under drought.

Breeding between elite, high-yielding, but drought-sensitive soybean genotypes, and low-yielding but drought-tolerant genotypes, has been a strategy to obtain offspring with higher tolerance to drought and more yielding (Fig. 2). For example, the genotype 'KS4895' (PI 595081) has high yield potential, but its BNF is sensitive to drought (Purcell et al., 2000); 'Jackson' (PI 548657), although drought-tolerant, has low yield potential. The crossbreeding between these genotypes resulted in several lineages like R01-581F and R01-416F that were selected among 600 progenies showing greater yield potential under water restriction (Chen et al., 2007a). These genotypes showed higher accumulation of N in shoots and BNF activity compared with the parental 'KS4895', in addition to higher yields under moderate drought (Sinclair et al., 2007).

The genotype R01-581F showed outstanding capacity to maintain physiological processes and BNF under water restriction, attributed to better osmotic adjustment, increase of the root system, and more effective translocation and metabolization of ureides (Alamillo et al.,

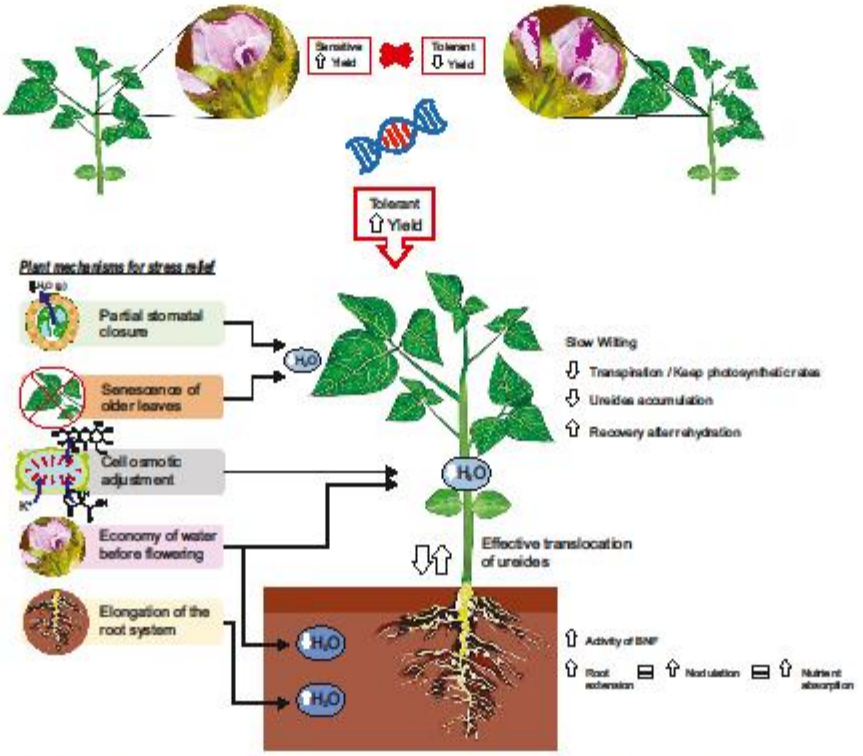

Fig. 2. Representation of plant-based strategies to relieve drought stress. Drought-tolerant but low-yielding genotypes can be crossed with drought-sensitive but high-yielding genotypes and produce offspring selected for tolerance and high yield. Arrows up indicate increase of the trait or process; arrows down indicate reduction of the trait or process.

2010; Cerezini et al., 2014; Cerezini et al., 2017; Cerezini et al., 2020). In a comparative study, the genotype 'Prima 2000' had superior performance under water restriction, with positive correlation between nodule size and shoot biomass, and yield (Fenta et al., 2014), showing that BNF-related traits had important effect on yield.

The soybean breed lines PI416937 and PI471938, expressing the slow-wilting phenotype under water restriction, have been used in breeding programs for development of drought-tolerant genotypes (Sinclair et al., 2008; Devi and Sinclair, 2013; Cerezini et al., 2016b; Engels et al., 2017). PI 416937 reduces the transpiration rates as the vapor pressure deficit (VPD) increase, resulting in water conservation in the soil (Sinclair et al., 2008) that can be used for longer periods. However, PI 471938 does not have the same physiological adjustment, since it does not show physiological advantages like early stomatal closure, and reduced transpiration under high VPD. Sadok et al. (2012) failed in identifying a physiological mechanism to explain the slow-wilting phenotype in PI 471938. Although the mechanisms are still unclear, this genotype maintains higher BNF activity, lower accumulation of ureides in leaves, and higher cell turgor under water restriction (Devi and Sinclair, 2013). Recently, Nogueira et al. (2020) reported that the greater capacity of PI 471938 to keep its BNF activity with soil drying could be explained by its smaller radii xylem elements, which are less subjected to cavitation, and thus maintaining water transport in the plant to support BNF activity, and ureids transportation from nodules to shoots. Using soybean plants contrasting for BNF sensitivity to drought, Pradhan et al. (2021) found strong correlation between decreases in transpiration rate of de-rooted plants due to clogging of larger-diameter xylem vessels exposed to a suspension of microspheres, and sensitivity of BNF to soil drying, suggesting that larger-diameter xylem vessels are disadvantageous in maintaining the BNF rate under drought. To our knowledge, strategies to improve tolerance to drought based on morphological traits of xylem vessels were never tried before, but these pioneer works have opened the discussion for a possible new trait to be considered in breeding programs.

The plant ability to recover from moderate drought after rehydration is also important (Rosas-Anderson et al., 2014; Cerezini et al., 2016b), and reflects on the plant yield potential (James et al., 2008; Prudent et al., 2015). Although effects of drought on soybean are well-documented, few studies have investigated the plant recovery capacity after rehydration. Genotypic variations in the ability to recover physiological processes with rehydration have been observed in soybean and peanuts (*Arachis hypogaea*) (James et al., 2008; Rosas-Anderson et al., 2014; Sulieman et al., 2015; Cerezini et al., 2016b). Mechanisms of drought tolerance and recovery depend on the maintenance of water in the tissues, water economy in the soil, or an integration of both (Rosas-Anderson et al., 2014). Genotypic differences between parental and progenies regarding the recovery capacity of BNF and plant transpiration after water restriction have been observed (Cerezini et al., 2016b). However, the genotypes that more recovered did not stood out for the full recovery of physiological processes after rehydration. Further research in this area is needed to elucidate the intricate mechanisms involved in the plant recovery process and contribute for the selection of soybean genotypes tolerant to drought. Host tolerance to drought implies in more capacity to keep fixing nitrogen for longer, attenuating the negative impacts of an early decrease of the BNF process on plant growth and yield (Sinclair and Nogueira, 2018).

Although a given trait might be promising for use in breeding programs for tolerance to drought, a single trait will probably not be enough to support BNF effectiveness under drought. Just as drought is considered a syndrome (Lawlor, 2002), i.e., a pool of responses to the stress, so is drought tolerance. Consequently, several traits and strategies should be taken into account to improve the success of BNF tolerance to drought.

4.2. *Plant growth-promoting rhizobacteria*

Plant growth-promoting rhizobacteria (PGPR) are free-living or associative microorganisms that provide a wide range of benefits to plants through several mechanisms, such as: BNF (Fukami et al., 2018); increased activity of nitrate reductase (El-Komy et al., 2003); synthesis of phytohormones like auxins, cytokinins, gibberellins (Fukami et al., 2017; Fukami et al., 2018), and ethylene (Strzelczyk et al., 1994); phosphate solubilization (Rodriguez et al., 2004); systemic acquired resistance, and tolerance to abiotic stress (Grover et al., 2010; Lakshmanan et al., 2017; Fukami et al., 2018; Rondina et al., 2020). Such microorganisms present several mechanisms that combined may favor the performance of crops under drought (Grover et al., 2010; Lakshmanan et al., 2017).

Azospirillum is the most currently studied PGPR and have been used as inoculant worldwide in several crops, and its use has impressively increased in Brazil and Argentina in the last years (Cassán et al., 2020). Recent studies have indicated beneficial effects of co-inoculation with *Azospirillum* and rhizobia in legumes, such as soybean and common bean (*Phaseolus vulgaris*) leading to early nodulation and increase of BNF rates, plant growth, and yield (Hungria et al., 2013; Chibeba et al., 2015; Cerezini et al., 2016a; Rondina et al., 2020). Benefits include protection against abiotic and biotic stresses (Fukami et al., 2018), alleviation of moderate drought on soybean (Cerezini et al., 2016a; Silva et al., 2019), and increase of soybean yield in sandy soils under moderate drought (Rondina et al., 2020). Beneficial effects of co-inoculation with PGPR were also observed under saline stress and nutritional limitation (Egamberdieva et al., 2013; Fukami et al., 2017). These contributions are attributed to extension of the root system promoted by *Azospirillum* due to production of phytohormones, such as auxins (Fukami et al., 2017); ethylene, cytokinins (Strzelczyk et al., 1994); gibberellins, abscisic acid (Cohen et al., 2009), and salicylic acid (Fukami et al., 2017). Besides stimulating root growth, phytohormones improve the root architecture for uptake of water and nutrients (Fukami et al., 2017; Fukami et al., 2018; Rondina et al., 2020). Moreover, *Azospirillum* is also related to the increased expression of enzymes involved in the cell detoxification process against reactive oxygen species (Fukami et al., 2017; Fukami et al., 2018). As observed, PGPR can be even more applied in soybean production systems to improve tolerance to drought. Not only *Azospirillum*, but also other microorganisms have been launched every year as inoculant or biofertilizer, some of them devoted to promote tolerance to drought, like *Bacillus aryabhattai* in cowpea (*Vigna unguiculata*) (Inthama et al., 2021). These strategies with different microorganisms must also be tested in soybean to check for their potential synergic effect on BNF and promotion of plant tolerance to drought (Fig. 3).

4.3. *Microbial secondary metabolites (MSM)*

Some plants and several microorganisms produce substances so

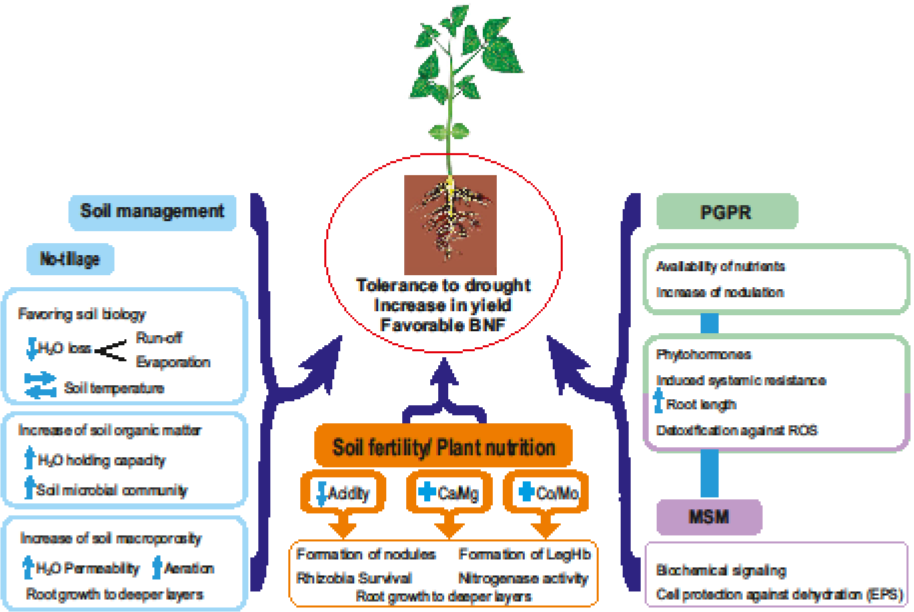

Fig. 3. Representation of strategies to relieve drought stress based on soil management, improvement of soil fertility, plant nutrition, inoculation with plant growth-promoting rhizobacteria (PGPR), and their microbial secondary metabolites (MSM).

named secondary metabolites, which are not essential for survival but improve its performance in the environment (O'Brien and Wright, 2011). In general, secondary metabolites are complex organic molecules synthesized in small amounts (O'Brien and Wright, 2011) and may act on defense, hormones, signaling in symbioses, microbial and plant growth-promoter, among others (Davies, 1992).

Rhizobia synthesize nodulation (Nod) factors that are MSM essential for communication and establishment of symbiosis with legumes at very low concentrations (Del Cerro et al., 2015; Gomes et al., 2019). These lipochitinoligosaccharides (LCOs) compounds are released in the surrounding environment and are recognized by specific receptors in the host plant cells, and triggers several genetic and metabolic events that lead to the root infection process (Cullimore et al., 2001; Kunert et al., 2016). Purified Nod factors may stimulate the symbiosis with rhizobia when applied on seeds or leaves of the host plant, increasing crop growth and yield (Skorupska et al., 2017). The addition of MSM at 0.1 mL L^{-1} of inoculant increased the number of nodules by 23.6% and the soybean yield by 4.8% compared with the treatment that received only *Bradyrhizobium* (Marks et al., 2013).

Early nodulation and more effective BNF are tools to deal with stressing conditions in the field, including drought (Cullimore et al., 2001; Cerezini et al., 2016a). A new generation of rhizobial inoculants containing MSM have proven effective in legumes by stimulating nodulation, lateral root development, increased mycorrhizal colonization, stimulation of the microbial community in the rhizosphere, tolerance to environmental stresses, and increase of crop yield (Marks et al., 2013; Del Cerro et al., 2015; Cerezini et al., 2016a; Gomes et al., 2019) (Fig. 3). As drought conditions impairs the signaling between the host plant and the corresponding rhizobia, enrichment of inoculum with Nod factors can be used as strategy to stimulate early nodulation, and, consequently, early BNF and N accumulation, and thus increase soybean tolerance to drought in the field.

4.4. *Soil management and fertility, and plant nutrition*

In addition to plant- and bacteria-related mechanisms to deal with drought, crop management strategies are also important tools to improve the plant performance. In the tropics, in low-fertility and acidic soils, no-tillage (NT) combined with crop rotation and liming creates conditions in the soil profile for roots to reach deeper layers to access water and nutrients, favoring the host plant and the N_2-fixing symbiont (Hungria and Vargas, 2000) (Fig. 3). Agricultural management, in addition to plant breeding, selection of more effective symbionts under drought, plant growth-promoting microorganisms and their MSM, are tools for improvement of the crop performance, leading to a more stable production system under moderate drought (Hungria and Vargas, 2000; Varshney et al., 2018).

The BNF process in soybean is generally more effective under NT than under conventional tillage (CT) (Barbosa et al., 2021), improving crop yield stability under drought due to more stable soil temperature and moisture during the crop cycle (Hungria and Vargas, 2000; Hungria et al., 2009; Franchini et al., 2012).

The CT based on plowing, chiseling, and harrowing degrades not only the physical and chemical soil properties, but also reduces microbial activity and diversity, especially in tropical environments (Babujia et al., 2010; Moraes et al., 2016). The exposition of the soil surface to solar radiation, wind, and impacts of rain drops causes not only physical disruption of soil aggregates, but also accelerates the water loss by evaporation. In addition, the constant mechanical disturbance of soil stimulates the oxidation of soil organic matter, worsening the scenario. Such environments make crops much more susceptible to short periods of rain shortage. On the other hand, the NT system improves the soil physical, chemical, and biological properties (Hungria et al., 2009; Franchini et al., 2012; Moraes et al., 2016). The possibility of roots to reach deeper soil layers and the increase of soil water holding capacity because of increase in soil organic matter under NT increases the amount of available water in the soil during shortage of rainfall.

In the tropics, high temperatures, drought, and soil acidity are limiting to the formation and functioning of root nodules in legumes. High temperatures and drought are the main causes of failure in nodulation, limiting the symbiosis and rhizobia survival in soil (Hungria and Vargas, 2000; Zilli et al., 2013). NT is efficient for conservation of soil organic matter (Hungria et al., 2009; Babujia et al., 2010), reduces temperature and increases the soil moisture, making a more favorable environment to the N_2-fixing microorganisms (Hungria and Vargas, 2000; Varshney et al., 2018). Not only availability of water but less oscillations of soil temperature are favorable to microbial processes, including BNF (Hungria and Vargas, 2000; Duval et al., 2017). The NT system increased the C and N microbial biomass in the soil and decreased the rate of C oxidation, promoting a positive balance of C into the soil in the long-term (Babujia et al., 2010; Hungria et al., 2009). In addition, NT reduces the water loss by evaporation and contributes for a more efficient use of water by plants (Huang et al., 2021) and reduces the critical period in which crops would be under water shortage.

Under temperate conditions, crop rotation with winter grasses were efficient in the production of mulching and led to an increase of soil organic matter that improved the cycling and reduced leaching of nutrients (Duval et al., 2017). Thus, the type of crop or cover crop in rotation influences the dynamics of soil organic matter depending on the composition of the crop residues, especially the C:N ratio. In tropical environments, crop rotation using tropical grasses, especially *Brachiaria* sp. associated with NT, has improved soil physical properties in comparison with the sole use of commercial crops after the main summer crop (Moraes et al., 2016). This conservative management has also increased the stability of the cropping system in years with uneven rainfall distribution (Franchini et al., 2012), in part because of better conditions for BNF (Hungria and Vargas, 2000).

Chemical limitations may also impair the BNF and increase the soybean susceptibility to drought. For a satisfactory nodulation and BNF in soybean, the soil pH must be above 5.2, which is easily reached with liming (Hungria and Vargas, 2000). In addition to improving soil pH and the availability of nutrients, mainly Ca^{2+} and Mg^{2+}, liming decreases the availability of toxic elements like Al^{3+} and Mn^{2+} in the profile of tropical soils.

Chemically and physically favorable soil conditions allow the root to grow into deeper layers, increasing the volume of soil to be exploited for water and nutrients (Fig. 3). Consequently, the conservative soil management with NT, crop rotations, and improvement of soil chemical properties mitigate the effects of drought and contribute for higher and more stable yields (Hungria and Vargas, 2000; Franchini et al., 2012).

Cobalt (Co) and molybdenum (Mo) are cofactors of enzymes and coenzymes involved in the BNF process. Mo takes part of the active site of nitrogenase, playing essential role in the BNF process (Campo et al., 2009). In addition, Co is involved in the formation of the leghemoglobin responsible for O_2 supply to the bacteroids in legume nodules (Hewitt, 1983). The soybean requirements of these micronutrients are generally low and actually applications of 2–3 g ha^{-1} of Co and 12–25 g ha^{-1} of Mo have been recommended in Brazil, either as seed treatment or as leaf spraying at early crop stages (Embrapa, 2020). Consequently, adequate supply of Co and Mo increases the effectiveness of BNF and allow plants to accumulate more N at early stages for later translocation to the formation of grains, resulting in higher yields (Campo et al., 2009). These benefits can be even higher due to co-inoculation with *A. brasilense* (Hungria et al., 2013; Galindo et al., 2017). In case of BNF constrains caused by drought at later stages of the plant cycle, a more effective N-fixation early in the cycle provided by sufficient plant supply with Co and Mo may help to alleviate the negative impacts on grain yields.

Several strategies can be applied to deal with drought stress on the soybean BNF. However, most of the studies have focused on a single strategy or trait to deal with drought. The combination of strategies taken together is probably more promising to improve the soybean BNF under drought. Plant selection based on genetic variability, genetic transformation, inoculation with plant growth-promoting rhizobacteria, use of microbial secondary metabolites, soil management, soil fertility and plant nutrition are among the strategies that should be taken together as much as possible as strategies to deal with drought. A summary of these strategies, the respective mechanisms and effects on plants are presented in Figs. 2 and 3.

5. Conclusions

Considering the importance of the soybean crop to the global economy and food security, and the impairment of BNF caused by drought, researches based on the host plant, microorganisms and their metabolites, and on soil management and plant nutrition are essential to increase the soybean tolerance to drought by improving the performance of the BNF and thus increasing the sustainability of the soybean cropping system. However, most of strategies do deal with drought as shown here have been adopted individually and may be not be as effective as when all available tools are considered in an integrated approach. Modern tools based on molecular biology, like differentially expressed genes, can help the assisted selection of plant genotypes and bacterial strains for tolerance to drought. Selection of PGPR, their metabolites as well as appropriate agricultural management (no-tillage, crop rotation, liming and nutrition), and plant morphological traits should be integrated strategies to improve soybean tolerance to drought by making the BNF process less sensitive to drought.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CAPÍTULO 2

INTERAÇÕES ENTRE GENÓTIPOS DE SOJA E ESTIRPES DE *Bradyrhizobium* SOB RESTRIÇÃO HÍDRICA

INTERAÇÕES ENTRE GENÓTIPOS DE SOJA E ESTIRPES DE *Bradyrhizobium* SOB RESTRIÇÃO HÍDRICA

RESUMO

O suprimento de nitrogênio para a soja em países da América do Sul se dá principalmente via Fixação Biológica de Nitrogênio (FBN). Porém, a seca prejudica o processo de FBN reduzindo a produtividade da cultura. A interação de genótipos tolerantes com estirpes de simbiontes tolerantes pode resultar em processos fisiológicos e FBN mais resilientes, com aumento da tolerância da cultura à seca. Neste trabalho, genótipos de soja contrastantes quanto à tolerância à seca foram inoculados com estirpes distintas de *Bradyrhizobium* e submetidos ao déficit hídrico induzido por polietilenoglicol (PEG) 6000 em condições hidropônicas para avaliar suas respostas fisiológicas, bioquímicas e moleculares. O genótipo sensível à seca BRS 284, inoculada com a estirpe CNPSo 06, teve maior acúmulo de prolina nos nódulos como ajuste osmótico quando exposta ao PEG 6000. No entanto, quando inoculada com CNPSo 1448 houve ajuste nas trocas gasosas, acúmulo de prolina nos nódulos, diminuição da atividade da superóxido dismutase (SOD), além da maior expressão de genes envolvidos na proteção celular e enzimas do estresse oxidativo no nódulo. No genótipo tolerante à seca PI 471938, a estirpe CNPSo 06 proporcionou maior ativação dos mecanismos de proteção celular, promovendo ajuste osmótico e aumento da atividade enzimática no nódulo, enquanto que a estirpe CNPSo 1448 promoveu ajuste nas trocas gasosas. A linhagem BR14-4760 originada do cruzamento entre BRS 284 × PI 471938 inoculada com CNPSo 1448 apresentou aumento do teor de prolina, diminuição da atividade da catalase (CAT) na folha e SOD nos nódulos. Quando inoculada com CNPSo 06, houve aumento na taxa fotossintética e redução na temperatura foliar, com diminuição da atividade de CAT foliar e *up-regulation* para todos os genes avaliados nos nódulos. A estirpe CNPSo 06 mostrou-se mais eficaz na indução de respostas de defesa da planta ao déficit hídrico.

Palavras-chave adicionais: *Glycine max*, restrição hídrica, transpiração, metabolismo antioxidante, expressão gênica.

Title

Interactions between soybean genotypes and *Bradyrhizobium* strains under water restriction

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Abstract

The supply of nitrogen to soybeans in South American countries is mainly based on the biological N_2 fixation (BNF). However, drought jeopardizes the BNF process and reduces crop yield. The interaction between tolerant genotypes with tolerant symbiont nitrogen-fixing strains may result in more resilient physiological and BNF processes, increasing the crop tolerance to drought. In this paper, soybean genotypes contrasting for drought tolerance were inoculated with different *Bradyrhizobium* strains and subjected to water deficit induced by polyethylene glycol (PEG) 6000 under hydroponic conditions to evaluate the plant physiological, biochemical, and molecular responses related to drought-tolerance. The drought-sensitive genotype BRS 284, inoculated with the CNPSo 06 strain had greater accumulation of proline in the nodules as osmotic adjustment when exposed to PEG 6000. However, when inoculated with CNPSo 1448 there was an adjustment in gas exchanges, proline accumulation in nodules, decrease in the activity of superoxide dismutase (SOD), in addition to greater expression of genes involved in cell protection and enzymes related to oxidative stress in nodules. In the drought tolerant genotype PI 471938, the CNPSo 06 strain provided greater activation of cell protection mechanisms, promoting osmotic adjustment and increased enzymatic activity in the nodule, whereas the CNPSo 1448 strain promoted adjustment in gas exchanges. The breed line BR14-4760 resulting from the crossing between BRS 284 × PI 471938 inoculated with CNPSo 1448 showed increase in proline concentration, decrease in catalase (CAT) activity in leaves and SOD in nodules. When inoculated with CNPSo 06, there was increase in the photosynthetic rate and reduction of leaf temperature, with decrease in leaf CAT activity and up-regulation for all assessed genes in the nodules. The CNPSo 06 strain showed to be more effective in inducing defense responses to water deficit.

Additional keywords: *Glycine max*, water restriction, transpiration, antioxidant metabolism, gene expression.

Introduction

Drought has been the main environmental factor that has caused major losses to soybean [*Glycine max* (L.) Merrill] yield worldwide (Zipper et al. 2016; Battisti et al. 2019; Zilli et al. 2020). In this sense, studies aiming at developing strategies to relieve the effects of drought have become emergent. In the last few years, the genetic improvement of soybean has focused on identifying genotypes with high yield potential and drought tolerance (Battisti and Sentelhas 2015; Nakagawa et al. 2018; Fried et al. 2019).

In South America, the main source of nitrogen (N) for soybean comes from the biological nitrogen fixation process (BNF) achieved by inoculating elite strains of *Bradyrhizobium* spp., allowing high yields with no need for N-fertilizer (Hungria and Mendes 2015). However, water stress directly compromises the BNF process even before transpiration and photosynthesis rate (Bellaloui and Mengistu 2015), limiting crop yield (Sinclair et al. 2007; Cerezini et al. 2016). Therefore, better understanding the soybean BNF sensitivity to drought and mechanisms of tolerance may contribute to select genotypes with more tolerant BNF and that have a better performance under water restriction conditions.

The genotype PI 471938 was identified as slow wilting and as having greater BNF tolerance to soil drying, since its BNF process supports drier soil conditions better than sensitive genotypes before the nitrogenase activity drops (Devi and Sinclair 2013; Riar et al. 2018; Cerezini et al. 2019). Thus, it can be used to transfer these characteristics in crosses with high-yielding commercial genotypesto obtain high-yielding genotypes tolerant to drought (Devi et al. 2014).

Water stress induces accumulation of reactive oxygen species (ROS) in different plant cell compartments that are toxic to the aerobic metabolism, and activates plant protection mechanisms such as antioxidant enzymes to keep the cell homeostasis (Mittler 2017; Choudhury et al. 2017). Therefore, for a better understanding of the mechanisms involved in the adaptation of plants to water stress, the evaluation of the activity of enzymes that protect against oxidative stress is indicated (Cruz de Carvalho, 2008). Superoxide dismutase (SOD) has been considered as the first defense line that catalyzes the dismutation reaction of anion radicals (You and Chan, 2015). Several SOD isoenzymes differ in their metal ion in the active center, like Cu, Zn, Mn, and Fe, being Cu/Zn-SOD the most common isoenzymes in plant cells (Amonova and Matniyazova, 2022). Several enzymatic pathways are responsible for scavenging H_2O_2, such as catalases and ascorbate peroxidase (Anjum et al., 2016).

The accumulation of osmoregulatory molecules such as proline (amino acid), trehalose (sugar), and betaine (amine) occurs in plant cells in response to water stress to maintain cell turgor pressure, preventing dehydration (Bartels et al. 2005; Seki et al 2007). Different capabilities to accumulate proline in response to drought have been reported among plant genotypes. Since greater accumulation suggests greater tolerance, it can be used as marker for identification of tolerant genotypes (Setter et al., 2012; Toscano et al., 2016).

The induction to water stress by adding high molecular weight polyethylene glycol (PEG) 6000 in nutrient solutions can be used for screening plant genotypes for tolerance to drought in greenhouse experiments (Sunaryo et al. 2016) or in culture medium for *in vitro* tests as it is a non-penetrating and non-toxic osmotic agent that reduces water potential in a similar way to soil drying (Saepudin et al. 2017). This osmotic agent can facilitate the investigation of interactions between soybean genotypes and *Bradyrhizobium* spp. strains to understand mechanisms that support the maintenance of the BNF process under water restriction.

Molecular biology approaches, such as identification of differentially-expressed genes, has been used to understand the mechanisms involved in the water stress response in the selection of drought-tolerant soybean genotypes (Fuganti-Pagliarini et al. 2017; Correa Molinari et al. 2021). However, most of gene expression studies on plant response to water stress have focused on roots and shoots, remaining unclear the mechanisms of the symbiosis response to drought in nodules.

This work aimed to evaluate the effect of induced water stress with PEG 6000 on physiological, biochemical, and molecular responses of soybean genotypes PI 471938, BRS 284, and their progeny BR14-4760 inoculated with different *Bradyrhizobium* spp. strains to seek for traits related to drought-tolerant BNF.

Material and Methods

Experiment I - previous evaluation on the genotype-strain interaction

The assay was performed in a greenhouse at Embrapa Soybean, Londrina, Paraná, Brazil, on February-March 2018. The treatments consisted of two standard strains: *Bradyrhizobium diazoefficiens* CNPSo 06 (= SEMIA 5080 = CPAC 7) and *B. elkanii* CNPSo 14 (= SEMIA 587 = BR 96), usually employed in commercial inoculants in Brazil (Hungria et al., 2006), CNPSo 56 (= SEMIA 5032 = USDA 110 = TAL 102 = ATCC 15034) classified as type strain (Delamuta et al., 2013), and two promising strains deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection #1054), in Londrina, State of Paraná, Brazil: *Bradyrhizobium* sp. CNPSo 1083 (= SEMIA 5082 = H 12 = CPAC 42), and *Bradyrhizobium* sp. CNPSo 1448. Each strain was grown in YM broth (Vincent 1970 with modifications Hungria et al., 2016) and inoculated on the seeds of two soybean genotypes, the parental BRS 284 (drought sensitive/high yield) and the progeny BR14-4760 (bred for slow-wilting), from crossing between BRS 284 × PI 471938 (known for showing the slow-wilting trait) (Devi and Sinclair, 2013). The inoculation corresponded to 1.2 $\times 10^6$ cells per seed and a non-inoculated positive N control receiving 80 mg of N as KNO_3 per pot every 10 days, resulting in a 2 × 6 factorial arrangement, with 4 replications. Plants were grown in modified Leonard jars (Vincent 1970; Howieson and Dilworth 2016) containing -sterilized substrate, consisting of a mixture of washed sand and crushed coal (2:1, v/v) with application of sterile N-free nutrient solution as needed (Yates et al. 2016). Jars were arranged completely randomized on the tables in the greenhouse and plants were grown up to 46 days and then evaluated. Shoots, roots, and nodules were dried at 60 ºC for determination of the respective dry weights (SDW, RDW, and NDW), number of nodules (NN) and specific mass of nodules (SMN). The N concentration in shoots was determined in sulfuric extracts by the green salicylate colorimetric method (Searle 1984) and then converted into shoot total N content based on the shoot dry biomass and expressed in mg per plant.

Experiment II - Determination of PEG 6000 concentration

After analyzing the effect of *Bradyrhizobium* strains on N accumulation in the soybean genotypes, the concentration of PEG 6000 solution needed to induce stress in soybean was determined. Soybean BRS 284 was sown and inoculated with 0.5 mL of the commercial inoculant - Total Nitro® containing *B. japonicum* SEMIA 5079 = CNPSo 07 and *B. diazoefficiens* SEMIA 5080 = CNPSo 06 with a concentration of 5×10^9 cells mL^{-1}, in Leonard jars containing sterilized substrate (as before) along with the application of N-free nutrient solution up to 34 days after emergence (DAE). Subsequently, at 35 DAE, the nutrient solution was replaced by PEG 6000 solutions in different concentrations, increasing from 0 to 12%, consisting of: 0; 1; 2.5; 3.5; 5; 6; 7.5; 10; 12, in 5 replications each. The effect of PEG on plants was evaluated by stomatal conductance (*gs*) as a physiological parameter by nondestructive analysis measured with a portable gas exchange device (LI-6400XT infrared gas analyzer IRGA - LICOR®, Lincoln, USA). Measurements were performed at 3, 6 and 10 days after PEG solution administration between 9:00 and 10:00 AM.

Experiment III - Transpiration test with de-rooted soybean shoots

The test was carried out with soybean genotypes with different sensitivity to drought to check for the evaporative capacity (Table 1). Plants were cultivated in a greenhouse, on January 2019, in plastic pots filled with 2 kg of substrate composed of a mixture of ravine soil, organic fertilizer and washed sand (3:2:2, v/v). At V3 developmental stage, plants were cut below the cotyledonary node and the de-rooted stems tips were quickly immersed in distilled water in a tray to avoid entrance air in the xylem vessels. Subsequently, stems were cut a second time with a sharp blade above the cotyledonary node while holding the stem under water. Then, five de-rooted shoots of each genotype were transferred to Erlenmeyer flasks containing 50 mL of distilled water so that the tip of the stem was immersed in the water and, sealed with plastic PVC film around the stem to avoid direct water evaporation. Preserved leaves were kept free to allow transpiration.

Table 1. Characteristics and pedigree of soybeans genotypes used in the test of evapotranspiration of de-rooted shoots

Soybean genotype	**Pedigree**	**Characteristic**
BRS 284	Mycosoy-45 × Suprema	Drought sensitivity/ high yield
NA 5909RG	-	Drought sensitivity/ high yield
PI 471938	-	Slow-wilting/ BNF drought tolerance
BR14-4760	PI 471938 × BRS 284	Unknown
BRR14-89152	PI 471938 × NA 5909 RG	Unknown

To determine the reference transpiration of each genotype, the transpiration rate was first measured providing only distillated water to the de-rooted shoots. After 30 minutes of stabilization, each flask containing the de-rooted shoot was weighed in a 2-figure scale and recorded. Weighing were performed every 20 min until stabilization. To check if transpiration rate was affected by different diameter of xylem vessels of each genotype (Nogueira et al. 2020), the shoots were then transferred to new Erlenmeyer containing a 0.01% agarose solution that clogs the smaller xylem vessels first, decreasing the transpiration rate (Pradham et al., 2021). The weights of the flasks supporting the de-rooted shoots were measured after 40 min of stabilization and then every 13 min for up to 130 min. The transpiration rate of each de-rooted shoot exposed to agarose was normalized by the ratio between the transpiration rate of the agarose-treated shoot and the average reference transpiration rate in water. Normalized transpiration rates (NTR) for shoots of each genotype were combined to calculate the rate of decrease in transpiration in NTR with time. A decrease in transpiration rate was interpreted as indicative of xylem vessels blockage by agarose, based on the study performed with microspheres by Pradhan et al. (2021).

During the evaluation of transpiration, air temperature and relative humidity were measured with a thermo hygrometer, which were 34.3 ºC (ranging from 30.4 – 34 .8 ºC) and 56.6% (ranging from 37 to 61%), respectively.

Experiment IV - induction of drought stress using PEG 6000

The treatments consisted in the same five strains used in experiment I, including a non-inoculated positive N control. The strains and the positve N control were combined with three soybean genotypes (BRS 284; BR14-4760; and PI 471938) and under two different water conditions: control without PEG addition and treatments with addition of PEG 6000 (6%) for five days from 34 days after the emergence (DAE), in a 3 × 6 × 2 factorial arrangement, with 4 replications.

Plants were grown in Leonard jars in completely randomized design. Seeds were inoculated with 0.5 mL per seed of each strain containing: CNPSo 06 - 3.8×10^9/ CNPSo 14 - 1.4×10^9/ CNPSo 56 - 4.8×10^9/ CNPSo 1083 – 4.9×10^9/ CNPSo 1448 – 2.6×10^9 by seed. The plants were grown up to 39 DAE in a greenhouse with air temperature and relative humidit of 28ºC (ranging from 24.2 to 32ºC) and 78% (ranging from 43 to 98%), respectively.

Up to 33 DAE, all plants were kept in nutrient solution without PEG 6000. At 34 DAE, during the final of the vegetative stage and at the beginning of flowering (R1), the water restriction was applied, in which the treatments had the nutrient solution replaced by a solution of PEG 6000 6% and the control plants continued to receive distilled water.

Gas exchanges

Gas exchanges were evaluated with a portable gas exchange device (LI-6400XT infrared gas analyzer (IRGA) - LICOR®, Lincoln, USA) with four days of exposure to PEG (38 DAE). Measurements were made in the central leaflet of the third fully expanded leaves from the plant apex of the main stem. The measurement conditions were: 366–390 µmol.mol^{-1} atmospheric CO_2, 500 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) supplied by LED lamps, 31–35 ºC leaf chamber temperature, and 45–58% relative humidity. The average equilibrium time for each set of measurements was 1 minute and 14 seconds (ranging from 40 seconds to 2 minutes and 31 seconds) performed between 9:20 and 11:30 am. The following parameters were measured: net photosynthesis rate (*A*; µmol CO_2 m^{-2} s^{-1}), stomatal conductance (*gs*; mol H_2O m^{-2} s^{-1}), internal CO_2 concentration in the substomatal cavity (*Ci*; µmol mol^{-1}), temperature of leaves (*Tleaf*; °C) and transpiration (*E*; mmol H_2O m^{-2} s^{-1}).

At the 5th day under water restriction (39 DAE) the shoots and nodulated roots were separated and immediately frozen in liquid N_2 and stored at -80 ºC for further analysis.

Biochemical analyses

Activities of the antioxidant enzymes superoxide dismutase (SOD; EC:1.15.1.1), catalase (CAT; EC:1.11.1.6), and ascorbate peroxidase (APX; EC:1.11.1.11) were measured in extracts obtained from the third fully expanded trefoil, without petioles, from all treatments. For nodules, the following treatments were selected: BR14-4760; PI 471938 and BRS 284 combined with strains CNPSo 06 and CNPSo 1448, under normal hydration conditions (control) or osmotic stress (addition of PEG 6000). For these assays, nodules were extracted from the crown root region.

Extractions for enzymatic analysis and SOD activity were performed according to Gianngiopolitis and Ries (1977), with modifications: extraction was performed by homogenizing approximately 200mg of tissue for 20 sec. in Ultra turrax® with 0.1% trichloroacetic acid (w/v), then the samples were submitted to centrifugation for 30 min. at 12.000 xg and 4ºC, and an aliquot of 250 microliters was used for the assay. Results were expressed in units (U) of SOD per milligram of protein quantified by the Bradford (1976) method, using bovine serum albumin as standard. CAT activity was evaluated as described by Havir and McHale (1987), and results expressed as micromoles per minute per milligram of protein. APX activity was evaluated according to Nakano (1981) optimized by Koshiba (1993), and results expressed as nanomoles per minute per milligram of protein.

Proline concentration in leaves and nodule was determined based on Bates et al. (1973), with modifications: for the preparation of the extract, approximately 0.15 g of fresh tissue (stored at -80º C) was homogenized in an Ultra turrax® for 20 seconds with 1 mL of 3% sulfosalicylic acid (w/v), then the samples were submitted to centrifugation for 30 min. at 12.000 xg and 4ºC, and an aliquot of 500 microliters was used for the assay.

Total RNA extraction, cDNA synthesis and quantitative RT-qPCR

For analysis of gene expression, only some treatments were selected, based on the following: strains that favored better nodulation in the interaction with the genotypes (experiment I); outstanding results in the analysis of gas exchanges (experiment IV). Nodules were detached from roots and finely ground in liquid N_2 with pestle and mortar. The total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of RNA were evaluated in a NanoDrop® ND1000 spectrophotometer (Uniscience) and the integrity was evaluated in 1% agarose gel stained with ethidium bromide. Following RNA extraction, genomic DNA was removed with DNAse I (Invitrogen). To verify the presence of genomic DNA, total RNA samples were amplified via conventional PCR with primers for β-actin gene (F: 5'-CCCCTCAACCCAAAGGTCAACAG-3' and

R: 5’-GGAATCTCTCTGCCCCAATTGTG-3’), along with standard genomic DNA – extracted from nodules using the DNeasy® Plant Mini kit (Qiagen) according to the manufacturer’s instructions. Samples containing only RNA did not show amplification. The high-quality total RNA was used to synthesize cDNA with SuperScript® III First-Strand kit (Invitrogen) according to manufacturer’s protocol. For cDNA validation, samples were amplified via conventional PCR with primers for β-actin gene along with standard genomic DNA.

Some genes encoding for drought-related responses in soybean were selected based on the available literature: aquaporin - 5Nod26, glutathione peroxidase - GPX6, glutamine synthetase – GS2, nodulin – Nod26, and polyubiquitin – SUBI-1. Genes involved in cell antioxidant metabolism were also selected: PRX, APX, CAT, and SOD. Selected genes and further information as name, main function, and gene model are shown in Table S.1.

The sets of primers for the qPCR targets were designed using primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) (Table S1) to obtain amplicons of 110–150 bp, based on gene sequences available in Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax) and NCBI (https://www.ncbi.nlm.nih.gov/).

The amplification efficiency for each primer set was determined using serial cDNA dilutions and calculated using the software LinRegPCR v.2012.0 (Ruijter et al. 2009). The specificity of the amplified products was evaluated by analyzing the dissociation curves. qPCR reactions were performed in biological and technical triplicate (n = 9) using the Platinum® SYBR Green® qPCR SuperMix-UDG with ROX kit (Invitrogen, Thermo Scientific) according to the manufacturer's instructions on a 7900HT Fast Real- Time PCR System (Applied Biosystems). The amplification conditions used were: initially 95 °C for 2 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. The dissociation curve was obtained with a cycle consisting of 95 °C for 15 s, 60 °C for 15 s and finally a period of 95 °C for 15 s. The gene expression level was determined using the formula 2-ΔΔct (Livak and Schimittgen 2001). The reference genes β-Actin and FYVE described by Marcolino-Gomes et al. (2015) were used for normalization, and the control plant samples without PEG 6000 were considered as calibrator to calculate the gene expression.

Statistical analyses

The dataset of experiments I, III and IV were submitted to tests of normality and homogeneity of variances followed by ANOVA with application of F test ($p \leq 0.05$). Once the effects of treatments or interactions between factors were detected, means were compared by Student-Newman-Keuls (SNK) test ($p \leq 0.05$). In experiment IV for RT-qPCR outputs, the *t* test was applied ($p \leq 0.05$). Pearson’s correlation coefficients ($p \leq 0.05$) were calculated using the AgroEstat software. To visualize the correlation matrix, a heatmap was constructed using the “ggcorrplot” package in RStudio for the following variables: Proline concentration, antioxidant enzymes (SOD, CAT and APX), and gene expression in the nodules.

Results

Plant biomass, nodulation, and N content in shoots

In this first set of analyzes, the objective was to evaluate the interaction among different *Bradyrhizobium* strains with the genotypes BRS 284 and BR14-4760 (Table 2S). For BRS 284, plants inoculated with strains CNPSo 06, CNPSo 14, and CNPSo 56 accumulated, respectively, 40%, 41% and 55% more shoot biomass than its BR14-4760 progeny. The inoculation with strain CNPSo 1083 reduced the shoot biomass of both genotypes compared with the commercial strains, and the same occurred for BRS 284 inoculated with CNPSo 1448 (Fig. 1A). For root biomass, BRS 284 had a reduction when inoculated with strains CNPSo 1083 and CNPSo 1448 compared with the commercial strains (CNPSo 06, CNPSo 14) (Fig. 1B).

Regarding to shoots N accumulation on BR14-4760, higher N content was observed in plants inoculated with strains CNPSo 06, CNPSo 14, and CNPSo 1448. For BRS 284, all strains resulted in more N accumulation compared with the positive N control and outstood in inoculated treatments with more N accumulation (20-54%) when compared with the progeny BR14-4760 (Fig. 1C). The number of nodules followed the same trend as N content for BR14-4760, however, strain CNPSo 06 stood out in BRS 284 (Fig. 1D). The nodule dry weight (Fig. 1E) folowed the same pattern as number of nodules, and N content for BR14-4760. For BRS 284, inoculation with strains CNPSo 06 and CNPSo 14 increased nodule weight by 23% and 36% respectively when compared with the progeny. Considering the specific mass of nodules (Fig. 1F), strain CNPSo 14 stood out among other strains inoculated in BRS 284, and with 36% increase over BR14-4760. Strains CNPSo 06, 56, and 1448 resulted in similar specific mass of nodules in both genotypes, whereas CNPSo 14 and 1083 presented lower results in the progeny compared with the parental.

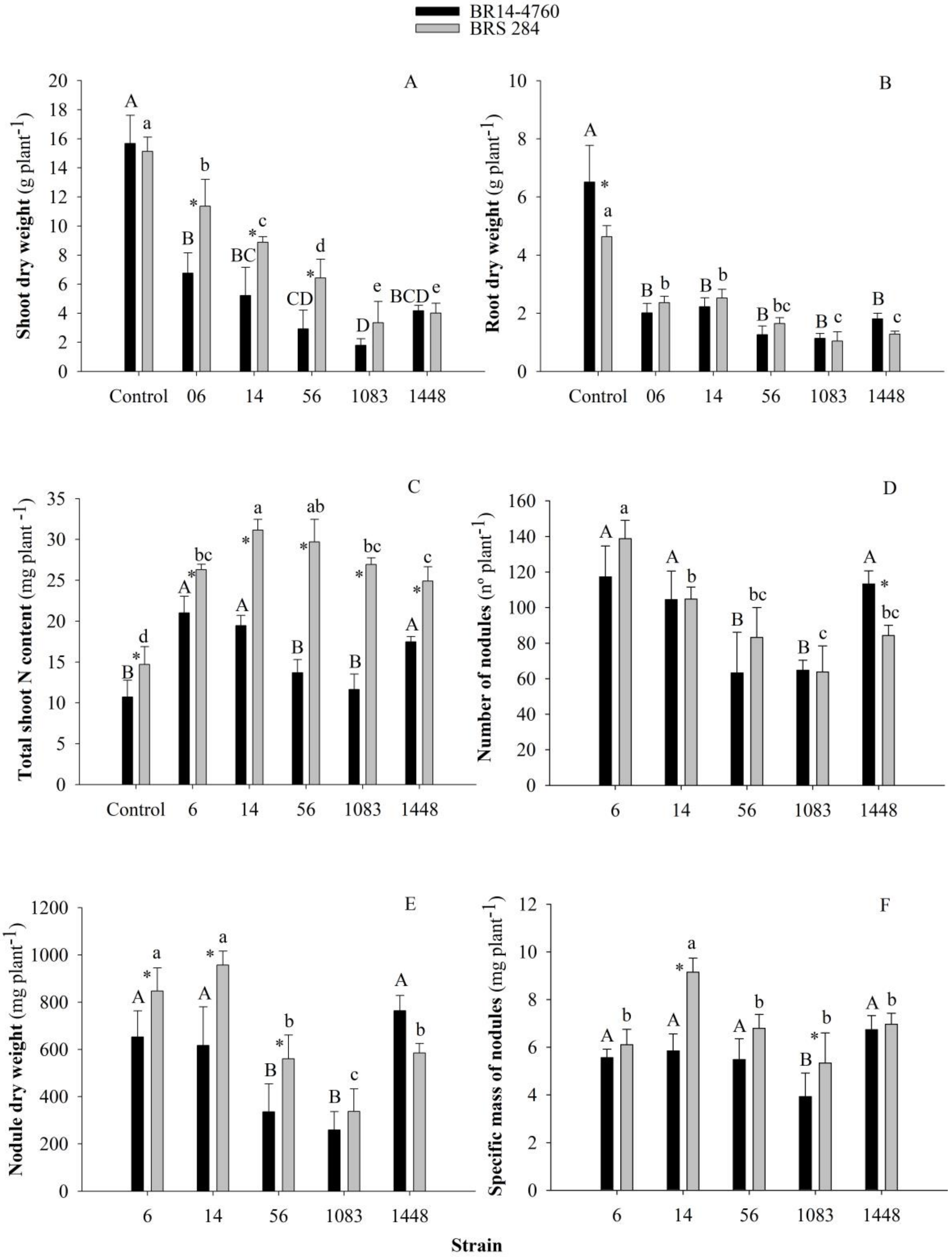


Fig. 1. Shoot dry weight (**A**), root dry weight (**B**), total shoot N content (**C**), number of nodules (**D**), nodule dry weight (**E**), and specific mass of nodules (**F**) according to soybean genotypes and inoculation with strains of *Bradyrhizobium*. Lowercase letters compare between strains within BRS 284; uppercase

letters compare strains within BR14-4760. * represent significant difference between genotypes within the same inoculation (SNK test $p \leq 0.05$). Vertical bars represent the standard deviation (n = 4).

PEG 6000 concentration

Stomatal conductance (*gs*) was assessed to determine the PEG 6000 concentration in which plants run osmotic stress, since stomata respond quickly to reduction of water availability. The *gs* values fell with increasing concentrations of PEG 6000 in the solution, irrespectively of the assessment times (Fig. 2). Since the average stomatal conductance with 10 days of exposure to PEG 6000 at a concentration of 6% was between 0.1 and 0.2 mol H_2O m^{-2} s^{-1}, considered as moderate stress (Flexas et al. 2004), that concentration was chosen for further studies.

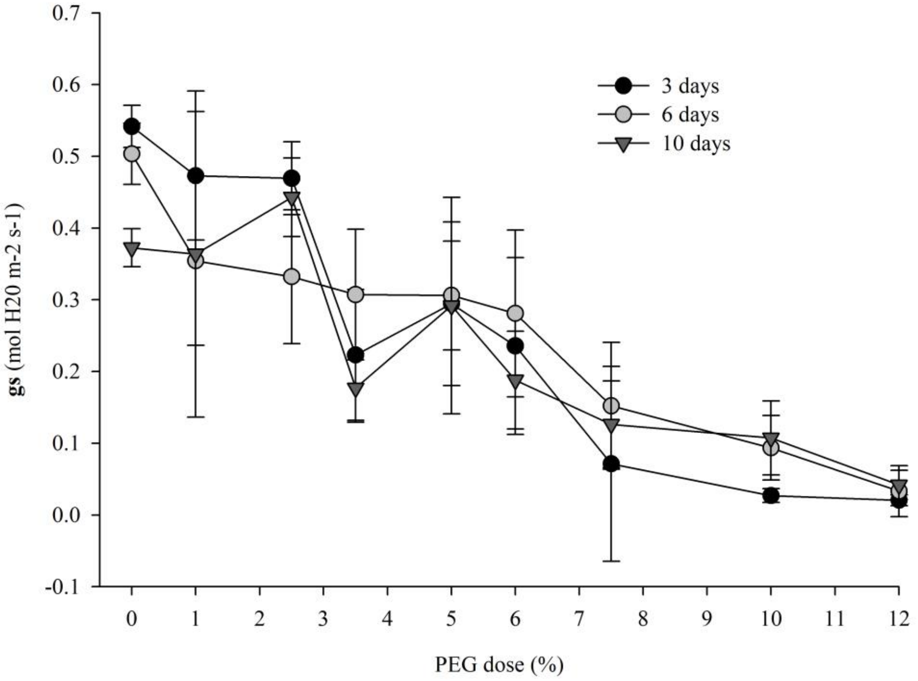


Fig. 2. Effect of increasing concentrations of PEG 6000 on leaf stomatal conductance (*gs*) of soybean cv. BRS 284 at three different times of exposure: 3 days, 6 days, and 10 days. Vertical bars represent the standard deviation (n = 5).

Transpiration Test

The exposure of de-rooted soybean shoots to 0.01% agarose dropped by half (0.5) the normalized transpiration rate after 53 min for most of genotypes. After 75 min, the NTR fell to below 0.3, except the progeny BR14-4760, which showed higher NTR values over time and droped to 0.5 only after 114 min of exposure to agarose (Fig. 3).

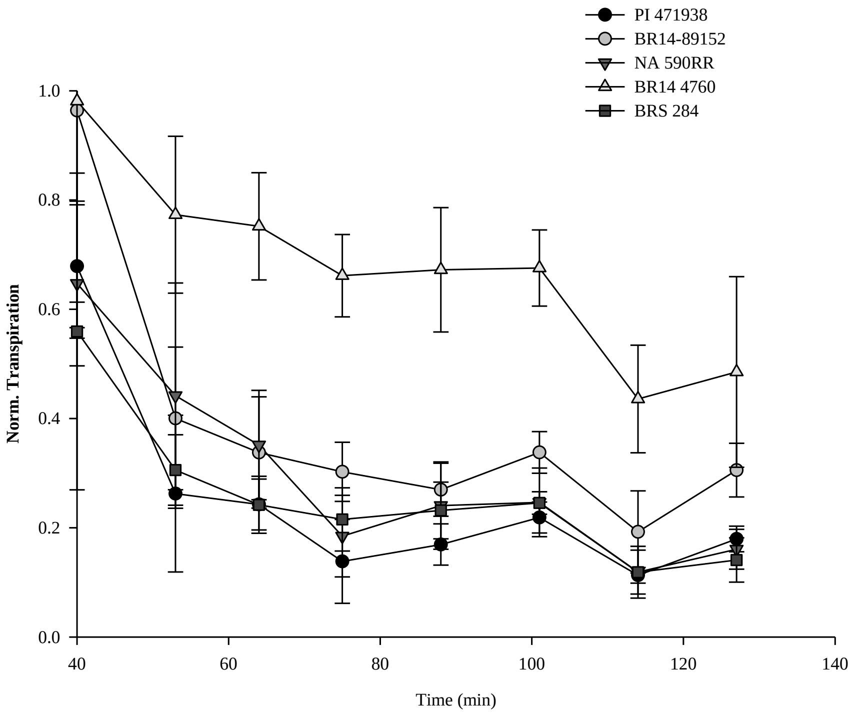
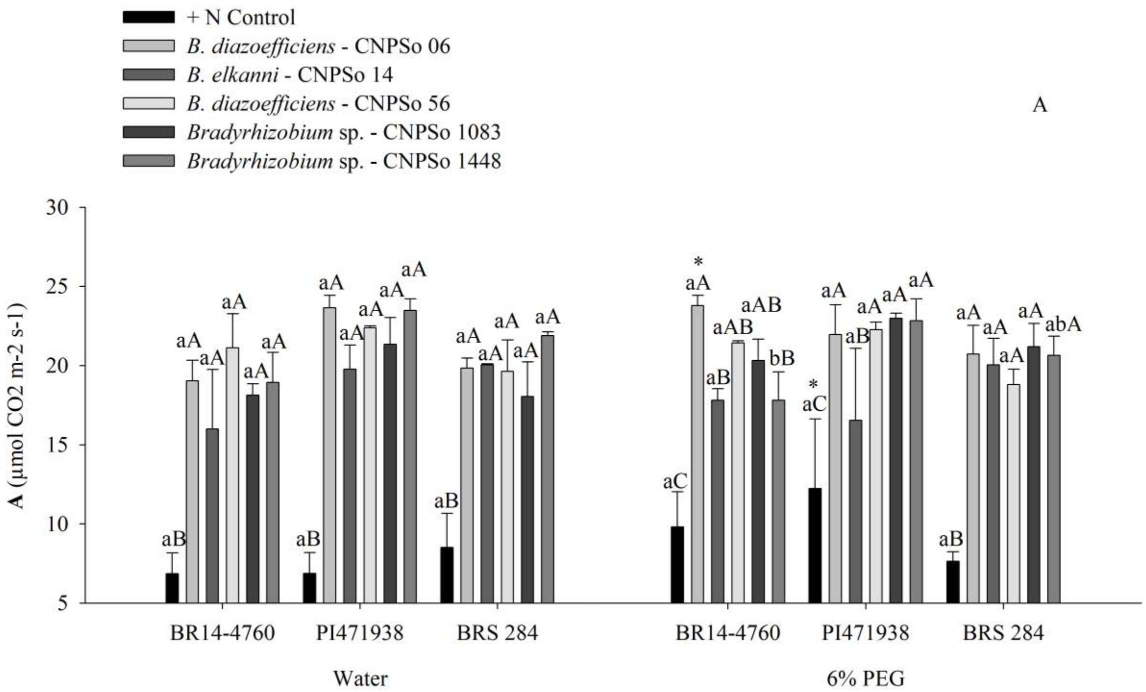


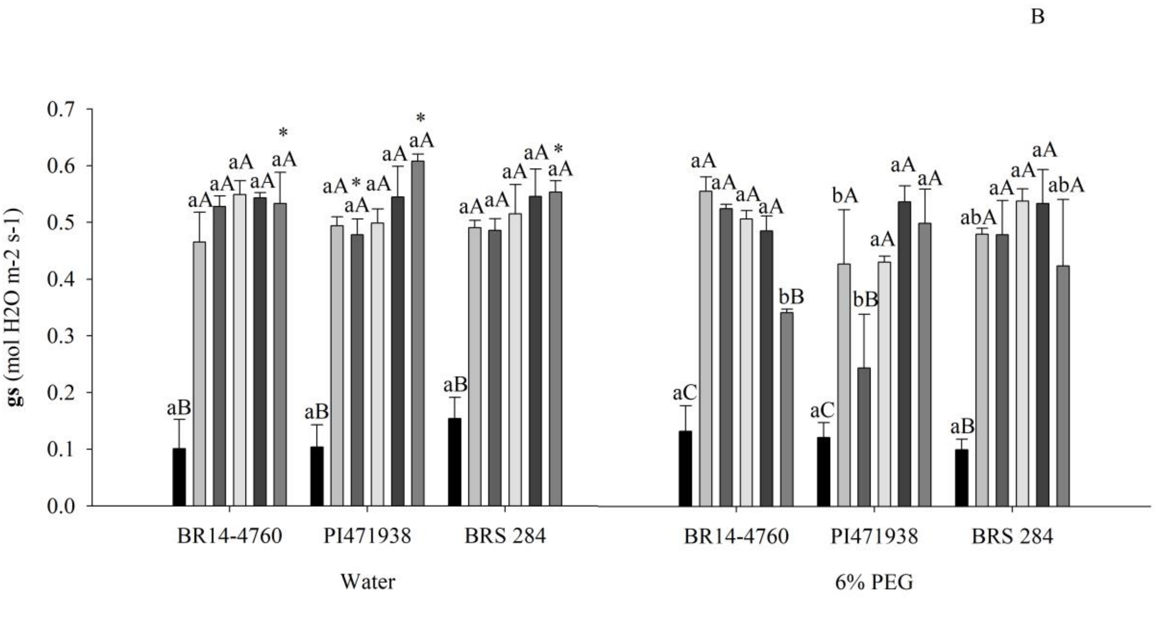
Fig.3. Normalized transpiration rate (NTR) of five soybean genotypes with contrasting traits for drought tolerance after exposure to a 0.01% agarose solution. Vertical bars represent the standard deviation (n = 5).

Simulation of water stress with 6% PEG 6000

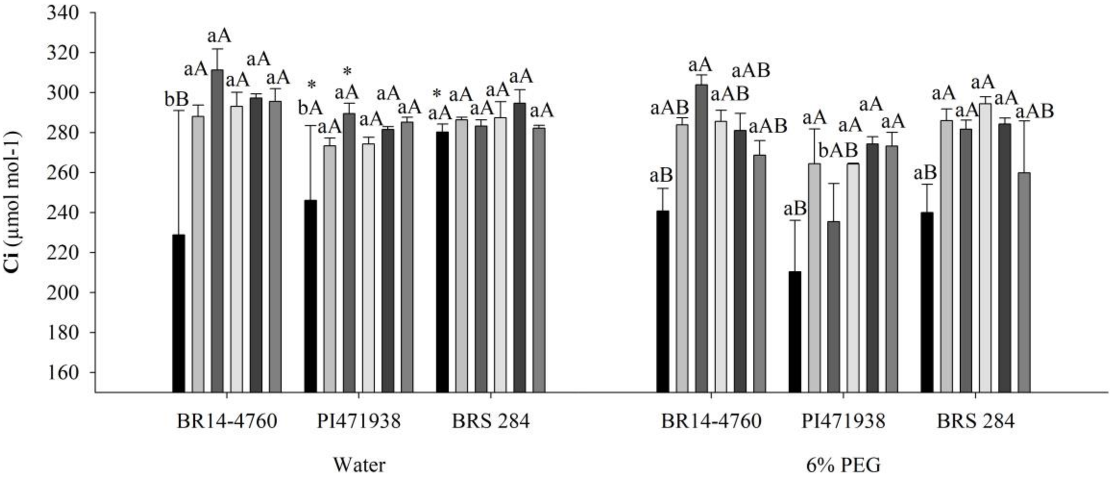
Gas exchanges

Photosynthetic rate (*A*), stomatal conductance (*gs*), and transpiration (*E*) were higher in inoculated plants compared with the +N control for the three soybean genotypes regardless of the water condition (Table 3S; Fig. 4), which evidences the positive effect of FBN on the physiological performance of soybean. When exposed to PEG 6000, the inoculation with the strain CNPSo 06 increased *A* in BR14-4760; conversely the strain *B. elkanni* CNPSo 14 decreased *A* in both PI 471938 and BR14-4760 (Fig. 4A). The most evident effect of strains on *gs* was observed in the PEG 6000 exposure, where inoculation with CNPSo 1448 decreased *gs* in genotype BR14-4760 and CNPSo 14 decreased it in PI 471938; exposure to PEG 6000 decreased *gs* in each genotype inoculated with CNPSo 1448 (Fig. 4B). Similar effect was observed in the +N control in wich the exposure to PEG 6000 decreased *Ci* by 15% in both PI 472938 and BRS 284 (Fig. 4C).

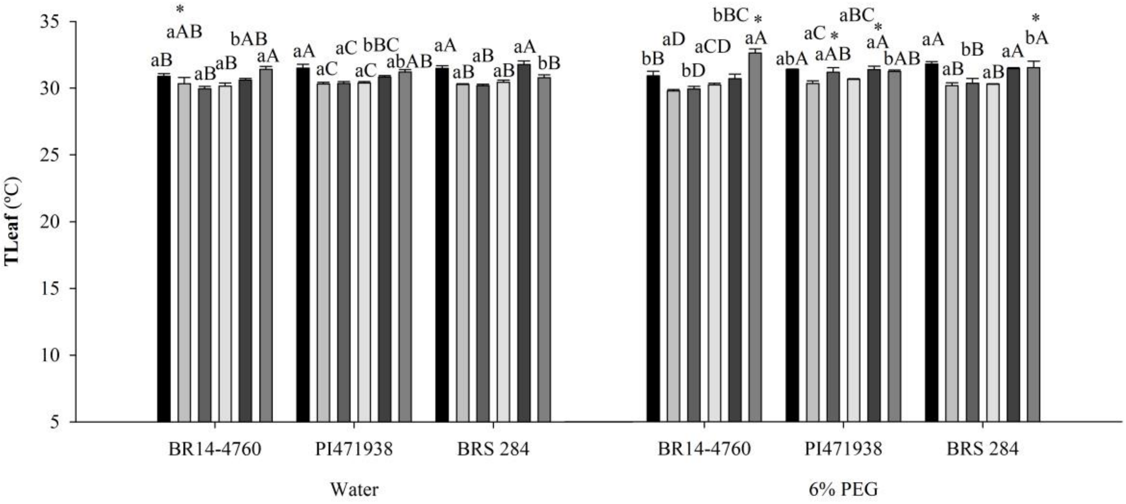

+ N Control
B. diazoefficiens - CNPSo 06
B. elkanni - CNPSo 14
B. diazoefficiens - CNPSo 56
Bradyrhizobium sp. - CNPSo 1083
Bradyrhizobium sp. - CNPSo 1448
A
A (µmol CO2 m-2 s-1)
30
25
20
15
10
5
aB aA aA aA aA aA
aB aA aA aA aA aA
aB aA aA aA aA aA
aC * aA aB aAB aAB bB
* aC aA aB aA aA aA
aB aA aA aA aA abA
BR14-4760
PI471938
BRS 284
BR14-4760
PI471938
BRS 284
Water
6% PEG


B
gs (mol H2O m-2 s-1)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
aB aA aA aA aA * aA
aB aA * aA aA aA * aA
aB aA aA aA aA * aA
aC aA aA aA aA bB
aC bA bB aA aA aA
aB abA aA aA aA abA
BR14-4760
PI471938
BRS 284
BR14-4760
PI471938
BRS 284
Water
6% PEG

C


Ci (µmol mol-1)
340
320
300
280
260
240
220
200
180
160
bB
aA
aA
aA
aA
aA
*
bA
aA
*
aA
aA
aA
aA
*
aA
aA
aA
aA
aA
aA
aAB
aA
aAB
aAB
aAB
aB
aB
aA
bAB
aA
aA
aA
aB
aA
aA
aA
aA
aAB
BR14-4760
PI471938
BRS 284
BR14-4760
PI471938
BRS 284
Water
6% PEG

D


TLeaf (ºC)
35
30
25
20
15
10
5
*
aAB
aB
aB
aB
bAB
aA
aA
aC
aC
bBC
aC
abAB
aA
aB
aB
aB
aA
bB
bB
aD
bD
aCD
bBC
*
aA
abA
aC
*
aAB
aBC
*
aA
bAB
aA
aB
bB
aB
aA
*
bA
BR14-4760
PI471938
BRS 284
BR14-4760
PI471938
BRS 284
Water
6% PEG

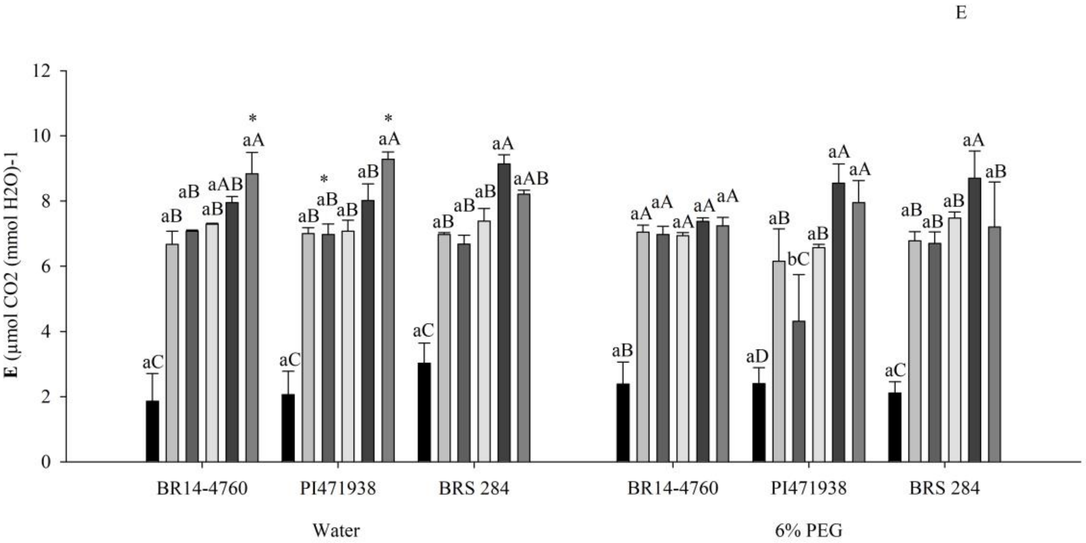


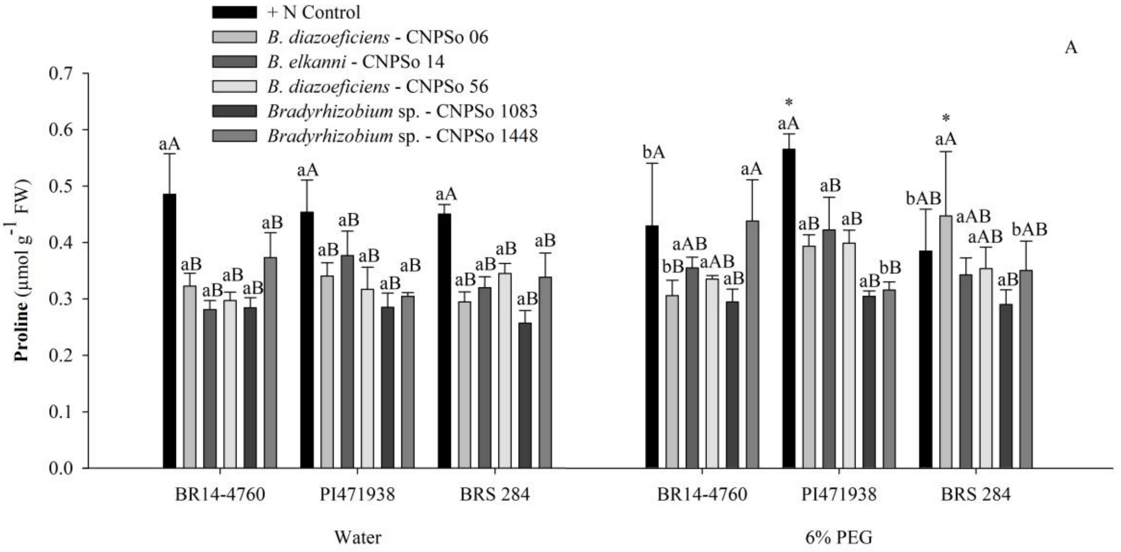
Fig. 4. Net photosynthetic rate (*A*) (**A**), stomatal conductance (*gs*) (**B**), internal CO_2 concentration in the substomatal chamber (*Ci*) (**C**), leaf temperature (*TLeaf*) (**D**), and transpiration (*E*) (**E**) of soybean inoculated with different *Bradyrhizobium* strains under regular water availability or exposed to 6% PEG 6000 during 4 days (38 DAE). Lowercase letters compare genotypes within the same strains and water condition. Capital letters compare strains within the same genotype and water condition. *Significant difference between water conditions for the same genotype and strain (SNK test $p \leq 0.05$). Vertical bars represent the standard deviation (n = 4).

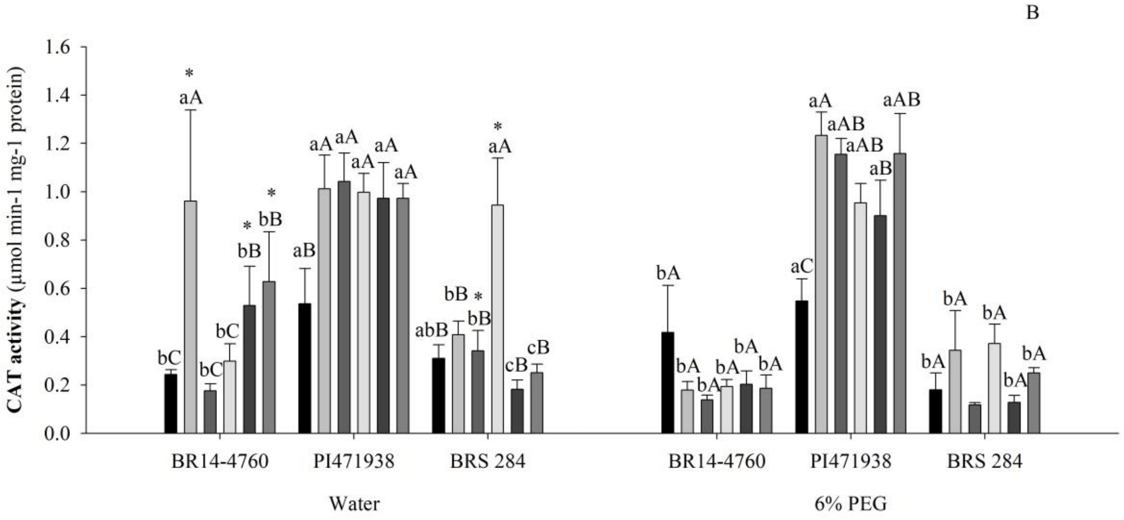
Tleaf increased in some treatments (within the same genotype and strain) due to exposure to PEG 6000, but in general there was little variation among genotypes and strains (Fig. 4D). The transpiration rate (*E*) was one of the variables mostly affected by strains within the same genotype and water condition, whereas genotypes litle varied withih the associated strain (Fig. 4E).

Proline concentration and enzyme activities in leaves

Under regular water supply, proline concentration was higher in the +N control plants compared with inoculated ones (Table 4S; Fig. 5A), which indicates a higher production of reactive oxygen species by the plant under mineral nutrition. Under osmotic stress, proline concentration increased by 20% in the PI 471938 control plants, higher than the other genotypes. For BR14-4760, inoculation with CNPSo 1448 increased proline in plants compared with CNPSo 06 and CNPSo 1083 strains. BRS 284 inoculated with CNPSo 06 strain showed higher values than the other strains and had even higher concentration than BR14-4760, with an increase by 35% (Fig. 5A). Generally speaking, greater CAT activity was recorded in the PI 471938 genotype, under normal water supply, suggesting a greater production of hydrogen peroxide resulting from the metabolic process in plants of this genotype.. For BR14-4760 and BRS 284, inoculation with CNPSo 06 increased CAT activity compared with the other strains, although CNPSo 56 also increased CAT activity in BRS 284.

Under osmotic stress, the CAT activity continued to be higher in PI 471938, espcially when inoculated with CNPSo 06 strain. BR14-4760 had significant reduction in CAT activity upon stress when inoculated with CNPSo 06 (-81%), CNPSo 1083 (-62%), and CNPSo 1448 (-78%) strains. The same occurred for BRS 284 plants inoculated with CNPSo 14 (-73%) and CNPSo 56 (-61%) strains (Fig. 5B).





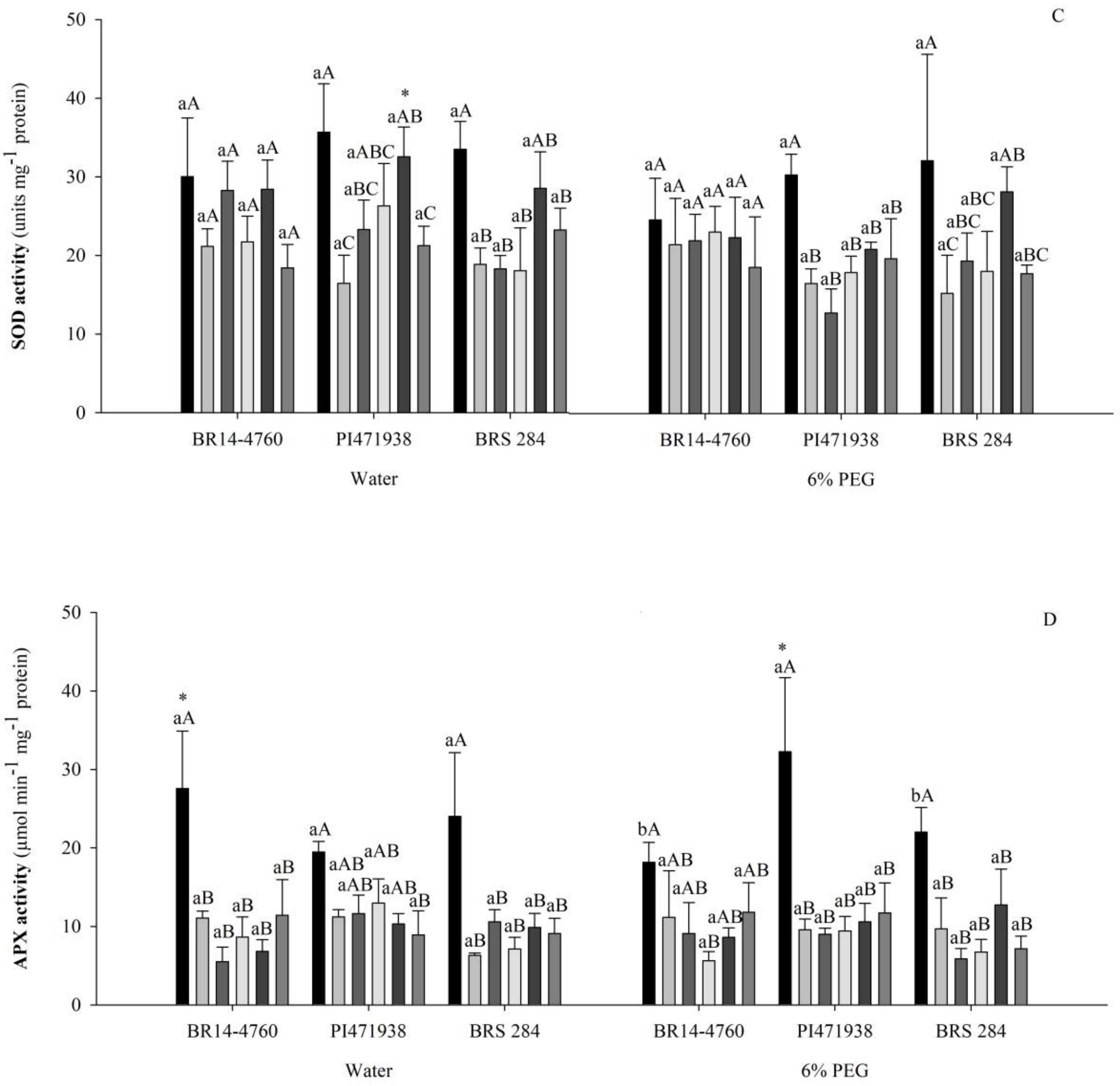


Fig. 5. Proline concentration (**A**), activities of catalase (CAT) (**B**), superoxide dismutase (SOD) (**C**), and ascorbate peroxidase (APX) (**D**) in leaves of soybean genotypes inoculated with different strains of *Bradyrhizobium* under normal water supply or osmotic stress induced by PEG 6000. Lowercase letters compare genotypes within strain in each water condition. Capital letters compare strains within genotype in each water conditions. *Significant difference between water conditions for the same genotype and strain (SNK test $p \leq 0.05$). Vertical bars represent the standard deviation (n = 4).

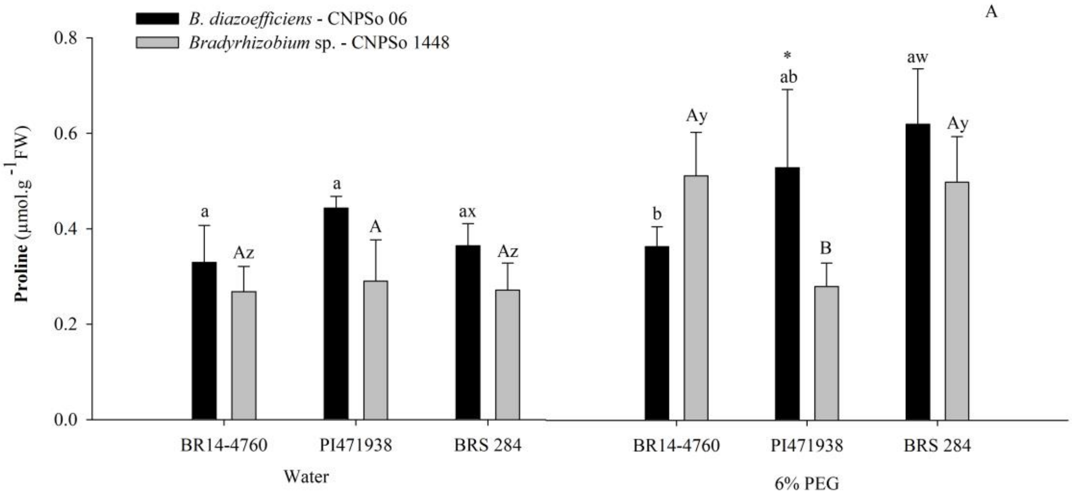
There was no effect of inoculation or water condition on SOD activity for the genotype BR14-4760. However, in general for both parentals the +N control had higher SOD activity compared with the inoculated plants, regardless of PEG 6000 exposure. Inoculation of BRS 284 with strain CNPSo 1083 increased SOD activity in both water conditions, but strain CNPSo 06 resulted the lowest activity under exposure to PEG 6000. Under nomal water supply, PI 471938 had higher SOD activity when inoculated with strains CNPSo 56, CNPSo 14, and CNPSo 1083, but the last two decreased SOD activity by 45%

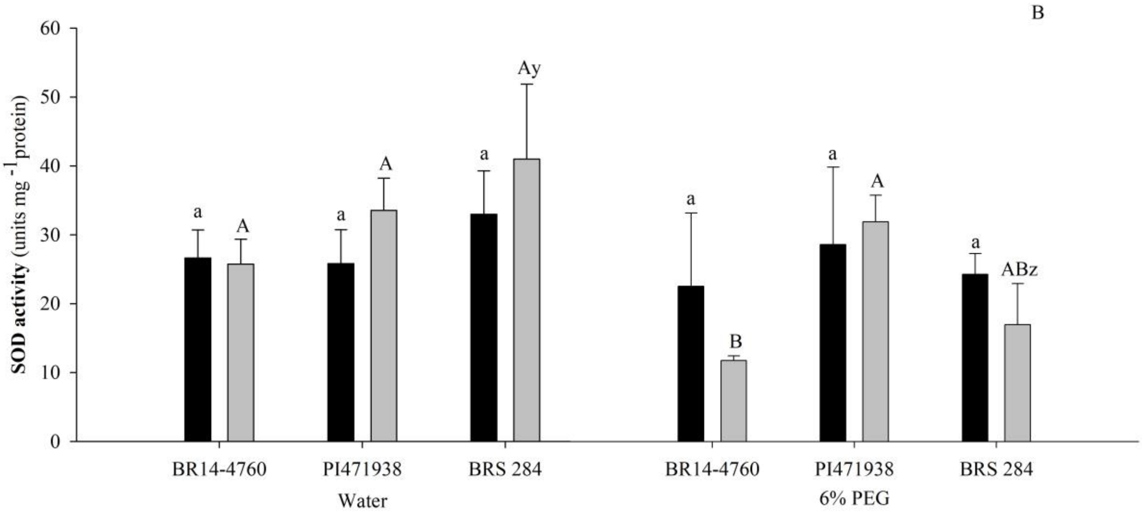
and 36%, respectively, under PEG 6000 (Fig. 5C).

Regardless of water condition and genotype, APX activity was lower in inoculated plants compared with +N control. Under osmotic stress, BR14-4760 inoculated with strain CNPSo 56 had the lowest APX activity compared with the other strains, whereas the +N control plants decreased it by 35%. The PI 471938 control plants had higher APX activity compared with the other genotypes, with a 41% increase in response to PEG 6000 (Fig. 5D).

Proline activity and enzyme activity in nodules

Under PEG 6000 exposure, BRS 284 inoculated with strains *B. diazoefficiens* CNPSo 06 and *Bradyrhizobium* sp. CNPSo 1448 had 42% and 46% more proline, respectively, standing out among the genotypes (Table 5S). The progeny BR14-4760 inoculated with strain CNPSo 06 had the lowest concentration, even under stress, whereas inoculation with CNPSo 1448 increased proline concentration by 48%. Conversely, PI 471938 inoculated with strain CNPSo 1448 had lower concentrations than the other genotypes inoculated wity *B. diazoefficiens* CNPSo 06 (Fig. 6A).





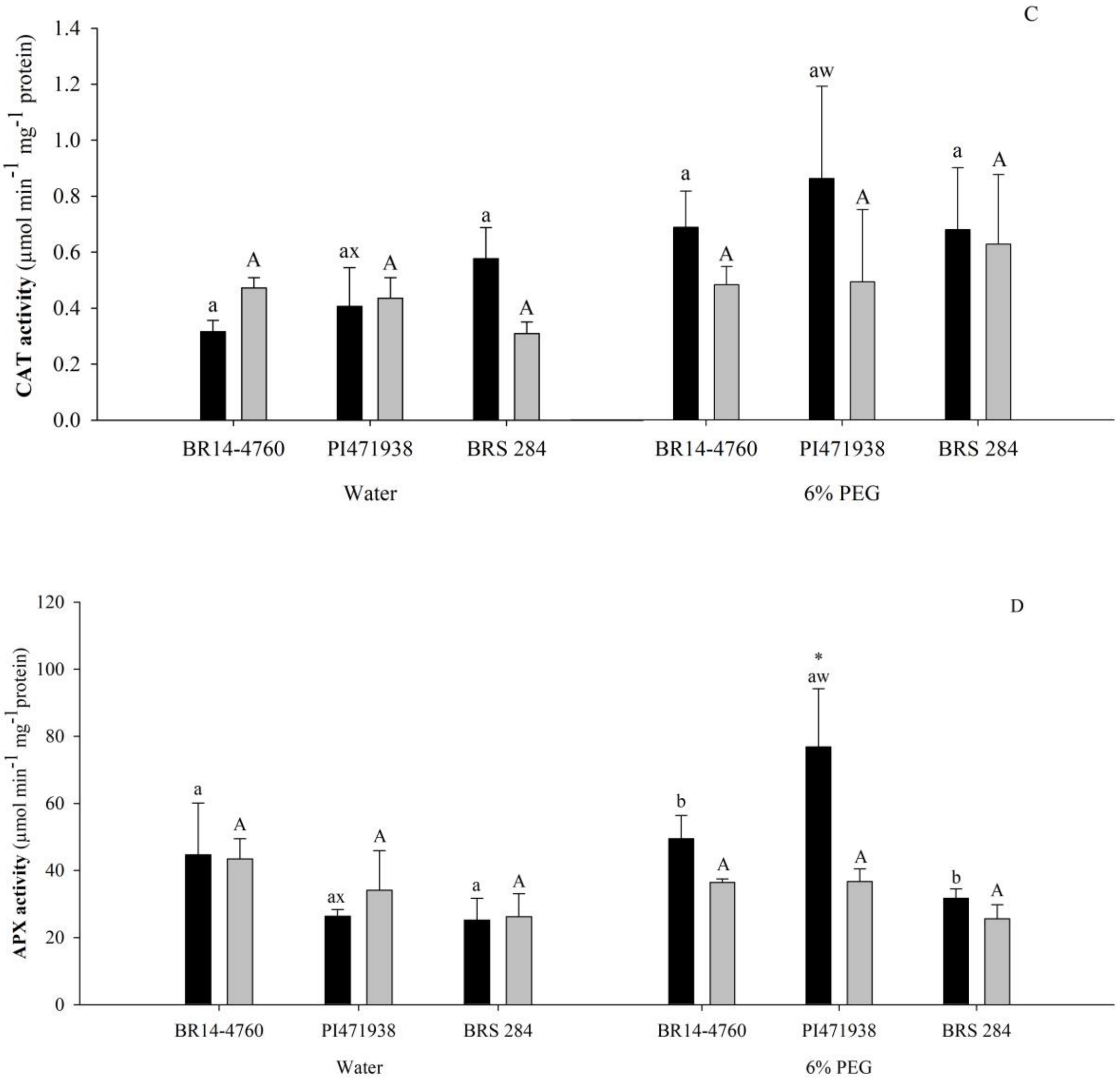


Fig. 6. Proline concentration (**A**), superoxide dismutase (SOD) (**B**), catalase (CAT) (**C**), and ascorbate peroxidase (APX) (**D**) in nodules of soybean genotypes inoculated with different strains of *Bradyrhizobium* under normal water supply or osmotic stress induced by PEG 6000. Lowercase letters compare between genotypes inoculated with *B. diazoefficiens* CNPSo 06 within each water condition. Capital letters compare genotypes inoculated with *Bradyrhizobium* sp. CNPSo 1448 within each water condition. *Significant difference between strains for the same genotype, within each water condition; "w" and "x" indicate significant differences between water conditions for the same genotype, inoculated with the strain CNPSo 06; "y" and "z" indicate significant differences between water conditions for the same genotype, inoculated with the CNPSo 1448 (SNK test $p \leq 0.05$). Vertical bars represent the standard deviation (n = 3).

Under water restriction, SOD activity in BR14-4760 inoculated with strain CNPSo 1448 was lower than the other genotypes and BRS 284 decreased activity by 58% in response to stress (Fig. 6B).

CAT and APX activities increased by 53% (fig. 6C) and 66% (Fig. 6D), respectively, in PI 471938 inoculated with strain CNPSo 06 in response to osmotic stress (Fig. 6D).

Relative gene expression

The expression of genes involved in N metabolism, regulatory processes, and antioxidant activity in soybean nodules were determined by RT-qPCR in response to simulation of water restriction. The relative expression of nine genes changed with exposure to PEG 6000 depending on the genotype and the symbiont strain (Fig. 7). For genotype BR14-4760 inoculated with *Bradyrhizobium diazoefficiens* CNPSo 06, osmotic stress up-regulated all genes in relation to nodules under normal water supply, with statistical significance for genes encoding aquaporin - 5Nod26 (1.47-fold) and nodulin – Nod26B (1.71-fold). When inoculated with the *Bradyrhizobium* sp. strain CNPSo 1448, only the polyubiquitin – SUBI-1 gene was significanlty up-regulated by 1.46-fold.

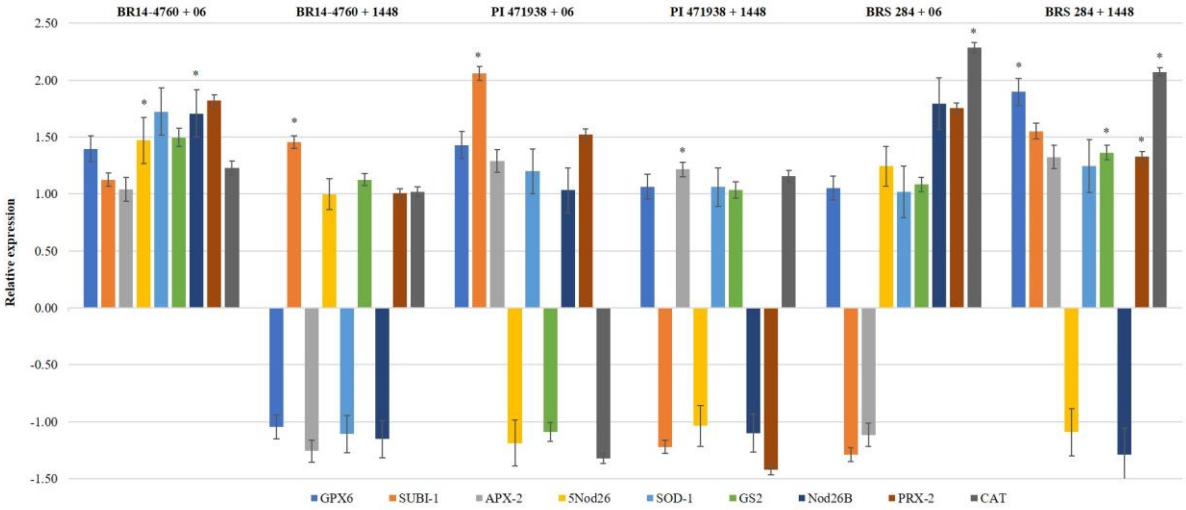


Fig. 7. RT-qPCR analysis of the relative expression of genes GPX6, SUBI-1, APX-2, 5Nod26, SOD-1, GS2, Nod26B, PRX-2 and CAT in nodules of soybean genotypes with different tolerances to drought, inoculated with *B. diazoefficiens* CNPSo 06 or *Bradyrhizobium* sp. CNPSo 1448, submited to osmotic stress by expostion to PEG 6000. Data were normalized to endogenous control (ß-Actin and FYVE). * Indicate statistically significant gene expression at α=5%, determined by the t-test. Vertical bars represent the standard deviation of three biological replicates, each with three technical replicates.

For PI 471938, the same up-regulation by 2.06-fold occurred when inoculated with strain CNPSo 06. However, when inoculated with strain CNPSo 1448 significantly increased up-regulation occured for APX-2 (1.22-fold). The down-regulations for SUBI-1, 5Nod26 and Nod26B were not statistically validated, whereas PRX-2 there was down-regulated by 1.42-fold, in contrast to th other genotypes that showed up-regulation irrespectively of the inoculated strain.

For BRS 284 inoculated with strain CNPSo 06 there was up-regulation for CAT by 2.29 times, wereas inoculation with strain CNPSo 1448 significanlty up-regulated GPX6 (1.90 x), GS2 (1.36 x) PRX-

2 (1.33 x), and CAT (2.07 x).

Correlation analysis between antioxidant enzymes and nodule gene expression

Most genes showed positive correlation each other, ranging from weak to moderate levels. There was no correlation between enzyme activities and its corresponding gene expression. The proline content showed positive correlation with CAT and negative correlation with SOD (Fig 1S).

Discussion

Plants have evolved several morphological, physiological, biochemical, and molecular traits to adapt to water stress (Santos et al. 2022). In this study we evaluated possible adjustments in leaf area, evapotranspiration, leaf gas exchanges, antioxidant mechanisms, and stress-induced gene expression in soybean genotypes with contrasting for BNF tolerance to drought and their interaction with *Bradyrhizobium* strains.

Nodulation and N_2 fixation in leguminous plants depend not only on the microsymbiont, but also on the host plant for an optimal interaction (Clúa, et al. 2018). The plant role on BNF implies in features that increase the N_2 fixation capacity, and therefore yield, but has often been neglected in breeding programs, as screening for plant traits to improve BNF is a laborious process (Sinclair and Nogueira 2018). Cerezini et al. (2020) observed that different strains of *Bradyrhizobium* spp. have different N_2 fixation capacity in soybean genotypes contrasting for drought tolerance regardless of water condition. This is in agreement with our study, in which plant biomass, nodulation, and N content varied with the interaction between BRS 284 genotype and its BR14-4760 progeny, and five *Bradyrhizobium* spp. strains under normal hydration conditions, highlighting CNPSo 06, 14 and 1448 strains for both genotypes.

To make easier the selection of drought-tolerant BNF genotypes, Pradhan et al. (2021) suggested the screening for tolerant genotypes based on the xylem vessel diameter. Larger diameter xylem vessels are vulnerable to cavitation, which stops the flow of N compounds from nodules to shoots and causes negative feedback on the BNF, while thinner vessels would keep the water flow (and transport of N compounds) for longer. Microscopic differences in xylem vessel diameters between PI 471938 (drought-tolerant BNF) and Hutcheson (drought sensitive BNF) indicated that the drought-tolerant genotype has predominance of thinner vessels in the xylem over the sensitive one (Nogueira et al., 2020).

In a study with 13 progenies derived from crossing between PI 471938 (tolerant) and Hutcheson (sensitive), Pradhan et al. (2021) exposed de-rooted shoots to microspheres of selected diameter to clog xylem vessels of larger diameters and identify the ones that keep the water flow for having thinner vessels. In our study, we found greater reduction in the normalized transpiration rate (NTR) of BRS 284 (parental 1) within 40 min in a solution with 0.01% agarose, whereas BR14-4760 (progeny) kept at least 50% of NTR for 114 min, what may indicate predominance of xylem vessels of smaller diameter promoting advantage under drought conditions. However, PI 471938 (parental 2) showed a similar behavior as BRS 284 after 53 min, suggesting that BR14-4760 progeny may have greater predominance of smaller diameter vessels compared with its parental. The maintenance of NTR by BR14-4760 under stress can be an efficient way thus allows plant recovery after the stress ceased (Cerezini et al. 2016).

Under water sufficiency conditions, inoculation with *Bradyrhizobium* spp. provided a better performance in the physiological parameters of the three genotypes evaluated, highlighting the effect of the strains as a growth-promoting microorganism. However, induction of water stress with PEG 6000 differentially affected gas exchange between soybean genotypes and also depended on the associated strain.

Plants inoculated with strains CNPSo 14, 06, and 1448 presented improved gas exchange traits in response to decrease in water availability. Cerezini et al. (2020) also reported improved gas exchanges with inoculation of *B. elkanii* SEMIA 5019 in the tolerant genotype R01-581F compared with the sensitive NA585RR under severe water restriction. In this study, PI 471938 inoculated with CNPSo 1448 run only a slight reduction in *E* and *gs* under stress, maintaining normal levels of gas exchanges. Conversely, inoculation with CNPSo 14 strain resulted in strong stomatal closure close to 0.2 mol H_2O m^{-2} s^{-1}, considered as moderate stress (Flexas et al. 2004), and strong reduction in photosynthetic rate, indicating a greater sensitivity of this interaction to water restriction with negative implications of the plant growth potential.

The maintenance of cell turgor pressure can contribute to keep physiological processes such as stomatal opening and photosynthesis, and is considered an effective mechanism of plant tolerance to drought (Zivcak et al. 2016). Accumulation of solutes is an important mechanism for protecting cell integrity by keeping positive turgor over a wider range of water potential (Setter 2012) and proline is considered one of the most important plants osmoregulator (Dong et al. 2019) where levels can be increased under water deficit. In this study, BRS 284 inoculated with strain CNPSo 06 had gas exchanges more resilient to osmotic stress possibly due to higher concentrations of proline in leaves, differently from the inoculation with CNPSo 1448 strain in which plants had lower proline levels compared to CNPSo 06 strain and underwent reduction in *gs* and increase in *TLeaf* as consequence of less transpiration due to stomatal closure.

The lower levels of proline and lower SOD and APX activities in soybean leaves from plants treated with *Bradyrhizobium* spp. obtained in this study, indicate the effect of inoculation in mitigating reactive oxygen species compared to nitrogen fertilization under regular hydration or water restriction.

Higher proline concentration in nodules can also protect from osmotic stress, as observed in PI 471938 inoculated with CNPSo 06 and in BR14-4760 inoculated with CNPSo 1448. Accumulation of proline as response to water stress (Zandalinas et al. 2017; Per et al. 2017; Fuganti Pagliarini et al. 2017) in more compatible inoculations with growth-promoting microorganisms can improve the plant tolerance to stress (Armada et al. 2014). In addition to playing role in the scavenging of free radicals, and osmoregulation, proline may serve as reservoir of carbon and nitrogen for cells after stress (Dutta et al. 2019), emphasizing its importance as a primary substrate to provide energy sources and carbon to resume the BNF process in nodules after stress relief.

Antioxidant enzymes like SOD, CAT, and APX are defense mechanism against reactive oxygen species (ROS), commonly generated by drought stress. SOD, which catalyzes the conversion of toxic O_2^- into peroxide (H_2O_2) is the first line of defense against ROS, and then CAT and APX come into action to convert H_2O_2 into H_2O (Sharma et al. 2012; Buezzo et al. 2019). In our study, inoculation of BR14-4760 and BRS 284 with CNPSo 1448 decreased SOD activity in nodules, as consequence of stress relief in the more compatible plant-microorganism interaction. In addition, SOD activity negatively correlated with proline in nodules, reinforcing the importance of this osmolyte for drought tolerance. As SOD activity produces H_2O_2, which is also toxic to the cells, increase in CAT activity must come just after to scavenge H_2O_2, as observed increase in BRS 284 inoculated with CNPSo 1448, as well as APX increase in PI 471938 inoculated with CNPSo 06. In leaves, CAT acts scavenging hydrogen peroxide generated in the electron transport chain and photorespiration (Barbosa et al. 2014). The highest CAT activity observed in PI 471938, regardless of inoculation and water conditions can be consequence of its higher photosynthetic rate than the other genotypes.

Osmotic stress promoted only slight variation in gene expression in nodules compared with normal water supply; most genes were upregulated whereas fewer where downregulated, depending on the interaction soybean genotype-*Bradyrhizobium* strain. The progeny BR14-4760 inoculated with CNPSo 06 had significant upregulation of aquaporin 5Nod26 and Nod26B genes, which act in the regulation of water transport (Barros de Carvalho et al. 2013), increasing hydraulic conductivity and facilitating water uptake from soil. This coincided with higher *A*, probably as consequence of higher *gs* and lower *Tleaf* that favored the maintenance of plant metabolism even under osmotic stress. The inoculation with CNPSo 1448 induced upregulation of Polyubiquitin (SUBI-1) gene under osmotic stress, suggesting activation in response to drought (Chen et al. 2016) to act in the activation of protein kinases, and in signaling in response to stress. Upregulation of SUBI-1 also occurred in PI 471938 inoculated with CNPSo 06 and may have favored cellular protection against oxidative damage. Fukao et al. (2011) reported higher expression of SUBI-1 in leaves under moderate stress resulting in reduction of ROS through activation of antioxidant enzymes.

Genes encoding for enzymes GPX-6, APX-2, and SOD-1, in addition to Nod26B, were downregulated in BR14-4760 inoculated with CNPSo 06, reinforcing the possibility of an initial control of drought by osmotic mechanisms instead of oxidative stress enzymes. It is plausive that plant response to stress starts with a try to keep water in the cells by means of osmotic regulations, whereas antioxidative responses would occur in the second step, when ROS are generated at higher levels due to stress. Conversely, PI 471938 inoculated with CNPSo 1448 showed upregulation of APX-2 gene with stress, as well as BRS 284 inoculated with CNPSo 06, with upregulation of CAT, although a corresponding increase in the corresponding enzymes was not detected. However, significant upregulation of GS gene in BRS 284 inoculated with CNPSo 06 may have favored the biosynthesis of proline, which concentration increased, evidencing the role of glutamate as precursor in the synthesis of proline as a mechanism of tolerance to abiotic stress (Roychoudhury et al. 2015). Accordingly, upregulation of GPX6, PRX-2, and

CAT genes is in agreement with higher CAT activity, which plays important role in detoxification of ROS induced by abiotic stresses, especially drought (Zandalinas et al. 2018).

Overall, this study strengthens the idea that soybean defense capacity, such as gas exchanges, osmotic adjustment, expression and activity of detoxifying enzyme activity induced by stress, is therefore dependent on the interaction between plant genotype and it associated *Bradyrhizobium* strain. Inoculation with CNPSo 1448 and CNPSo 06 strains generally triggered a differential expression of genes involved in cell protection and detoxifying enzymes becoming plant more resilient to face the stressing condition, even the drought-sensitive BNF genotype BRS 284. PI 471938 inoculated with strain CNPSo 06 had improved mechanisms of cell protection against water deficit, whereas BR 14-4760 progeny had higher photosynthetic rate and lower leaf temperature. Based on these improved traits, in addition to higher NTR, combination of BR14-4760 with the strain of *Bradyrhizobium* CNPSo 06 stands out as the best combination do deal with water restriction. However, evaluations of the performance of this interaction in the field are necessary, in addition to other mitigation strategies, such as co-inoculation with *Azospirillum brasilense*, the addition of secondary metabolites, supplementation with Cobalt and Molybdenum, which may favor the development and productivity of soybean under conditions of drought.

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Supplementary Information

Table 1S. Sequences of primers flanking genes used for analysis of relative gene expression by RT-qPCR with their respective efficiency values.

Gene	**Description**	**Gene Model**	**Primer sequence (5'-3')**	**Efficiency (%)**
5Nod26	Aquaporin proteins acting as facilitator of water transport	Glyma08g12650	F - CATAGCTGGAGCATGGGTA R – ACGGCCTTTGAGGAAAGAAG	88
GPX6	Cell protection against oxidative damages (glutathione peroxidase)	Glyma05g37900.1	F - AGATGCTGCTGCAATTTCCT R – ATCACTGGCATGGCTAAAGG	83
GS2	Metabolism of N compounds (glutamine synthetase)	Glyma15g10890.2	F - TGCCCAGTTATTGGTTGTCA R – AGAAAATCCGAGGGGACACT	76
Nod26B	Aquaporin-encoding genes found in nodulation	Glyma19g22210	F - CCTTTAGCCGCTTGTCTCAT R – CTGGAAGCCAATAGGCTGAT	75
SUBI-1	Polyubiquitin acting primarily as regulator of gene expression in responses to stress and defense under drought and flooding	Glyma20g27950.1	F - TGAATTGGCTAGAGGGGACTT R – CACCATCCTTCACACAACTCA	99
SOD-1	Cell protection against oxidative damages (Copper/zinc superoxide dismutase).	Glyma03G242900.1 Glyma19G240400.1*	F - TTTGGTTAGGTCCGGTCTGT R – AGGATAACCACCAACACATACT	79

CAT	Cell protection against oxidative damages (catalase - peroxidase)	Glyma.14G223500.1	F - GAAGTCTGTGGCACTGTAAT R – CAAATGAACGGAGAATCAT	78
APX-2	Cell protection against oxidative damages (L-ascorbate peroxidase 2, cytosolic)	Glyma.12G073100.1	F - CCCTTTGTAGTATGTGACCA R – GCCAATCTAACGGTCCTAAA	84
PRX-2	Cell protection against oxidative damages - detoxifying peroxides (peroxiredoxin)	Glyma.09G192800.1	F - CCCTTGAATCCTCTTGTTGGC R – CTTCCCAGAGCACACAACAC	78
B-Actin	Reference Gene	Glyma.15g05570	F - GAGCTATGAATTGCCTGATGG R – CGTITCATGAATTCCAGTAGC	90
FYVE	Reference Gene	Glyma.13g17500	F - TTCTGTCTTCTGCAAGTGGTG R – GATCCCTCATCCATACATTTCAG	92

Table 2S. Shoot dry weight (*SDW*), root dry weight (*RDW*), Nodule number (*NN*), nodule dry weight (*NDW*), specific mass of nodules (*SMN*), and total shoot N content (TSNC) in soybean genotypes differing for BNF tolerance to drought, inoculated with different *Bradyrhizobium* strains

Factors	***SDW***	***RDW***	***NN***	***NDW***	***SMN***	***TSNC***
Soybean Genotypes (SG)	g plant^{-1}	g plant^{-1}	per plant	mg plant^{-1}	mg plant^{-1}	g plant^{-1}
BR14-4760	6.1 b	2.2 a	93 a	526 b	5.5 b	15.7 b
BRS 284	8.2 a	2.5 a	95 a	657 a	6.9 a	25.6 a
Inoculation with BNF (BNF)						
+N Control	15.4 a	5.6 a	-	-	-	12.7 d
B. diazoefficiens CNPSo 06	9.1 b	2.2 bc	128 a	749 a	5.8 b	23.6 ab
B. elkanni CNPSo 14	7.0 c	2.4 b	105 b	787 a	7.5 a	25.3 a
B. diazoefficiens CNPSo 56	4.7 d	1.4 cd	73 c	448 b	6.1 b	21.7 bc
Bradyrhizobium sp. CNPSo 1083	2.6 e	1.1 d	64 c	298 c	4.6 c	19.3 c
Bradyrhizobium sp. CNPSo 1448	4.1 de	1.5 cd	99 b	675 a	6.8 ab	21.2 bc
ANOVA						
SG	**	*ns*	*Ns*	**	**	**
BNF	**	**	**	**	**	**
SG × BNF	*	**	*Ns*	**	*	**
CV (%)	23.16	25.72	19.87	21.67	15.04	10.42

Inoculation with BNF (BNF): +N Control (addition of N); inoculation at sowing with *Bradyrhizobium diazoefficiens* CNPSo 06; *Bradyrhizobium elkanni* CNPSo 14; *Bradyrhizobium diazoefficiens* CNPSo 56; *Bradyrhizobium* sp. CNPSo 1083; *Bradyrhizobium* sp. CNPSo 1448. Means (n = 4) followed by the same letter do not differ (*ns*) by SNK test at *$p \leq 0.05$ and **$p \leq 0.01$.

Table 3S. Net photosynthetic rate (*A*), stomatal conductance (g_S), internal CO_2 concentration in the substomatal chamber (*Ci*), transpiration (*E*), and temperature of leaves (*TLeaf*) of soybean genotypes inoculated with different *Bradyrhizobium* strains under normal water supply of osmotic stress induced by PEG 6000.

Factors	***A***	***gs***	***Ci***	***E***	***TLeaf***
Soybean Genotypes (SG)	µmol CO_2 m^{-2} s^{-1}	mol H_2O m^{-2} s^{-1}	µmol mol^{-1}	µmol CO_2 (mmol $H_2O)^{-1}$	ºC
BR14-4760	17.6 b	0.438 a	281 a	6.5 a	30.6 b
PI 471938	19.7 a	0.415 a	264 b	6.4 a	30.9 a
BRS 284	18.1 b	0.441 a	280 a	6.7 a	30.9 a
Inoculation with BNF (BNF)					
+ N Control	8.6 c	0.118 c	241 b	2.3 c	31.3 a
Bradyrhizobium diazoefficiens CNPSo 06	21.5 a	0.484 ab	280 a	6.8 b	30.2 c
Bradyrhizobium elkanni CNPSo 14	18.3 b	0.456 b	284 a	6.4 b	30.3 c
Bradyrhizobium diazoefficiens CNPSo 56	20.9 a	0.505 ab	283 a	7.1 b	30.4 c
Bradyrhizobium sp. CNPSo 1083	20.3 a	0.531 a	285 a	8.3 a	31.1 b
Bradyrhizobium sp. CNPSo 1448	20.9 a	0.492 ab	277 a	8.1 a	31.5 a
Water Condition (WC)					
Control	18.1 a	0.455 a	282 a	6.7 a	30.7 b
PEG	18.8 a	0.407 b	268 b	6.3 b	30.9 a
ANOVA (*F probability*)					
SG	**	*ns*	**	*ns*	**
BNF	**	**	**	**	**
WC	*ns*	**	**	**	**
SG × BNF	*ns*	**	*ns*	*	**
SG × WC	*ns*	*ns*	*ns*	*ns*	*ns*

BNF × WC	*ns*	**	*ns*	*ns*	**
SG × BNF × WC	*ns*	*	*ns*	*ns*	**
CV (%)	13.08	13.98	7.28	11.39	1.01

Soybean Genotypes: SG = BR14-4760 – progeny of PI 471938 x BRS 284; PI 471938 – drought tolerance; BRS 284 – drought sensitive.
Inoculation with BNF (BNF): Positive Control – (addition of N); inoculation at sowing with *Bradyrhizobium diazoefficiens* (strain SEMIA 5080/ CNPSo 06); *Bradyrhizobium elkanni* (strain CNPSo 014); *Bradyrhizobium diazoefficiens* (EUA) (strain CNPSo 056); *Bradyrhizobium* sp. (strain CNPSo 1083); *Bradyrhizobium sp.* (strain CNPSo 1448).
Water Condition: Control – Absence of application of PEG (Polyethylene glycol) 6000 solution; PEG- Application of PEG (Polyethylene glycol) 6000 solution.

Table 4S. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and concentration of proline in soybean genotypes inoculated with different *Bradyrhizobium* strains under normal water supply or under osmotic stress induced by PEG 6000.

Factors	**SOD**	**CAT**	**APX**	**Proline**
Soybean Genotypes (SG)	mg protein^{-1}	µmoL min^{-1}	mg protein^{-1}	µmol g^{-1} FW
BR14-4760	22.4 a	0.3251 b	11.3 b	0.3497 a
PI 471938	22.8 a	0.9569 a	13.1 a	0.3726 a
BRS 284	22.6 a	0.3169 b	10.9 b	0.3473 a
Inoculation with BNF (BNF)				
+N Control	31.1 a	0.3381 d	23.9 a	0.4608 a
Bradyrhizobium diazoefficiens CNPSo 06	18.3 c	0.6895 a	9.84 b	0.3502 b
Bradyrhizobium elkanni CNPSo 14	20.7 c	0.4905 c	8.61 b	0.3492 b
Bradyrhizobium diazoefficiens CNPSo 56	20.9 c	0.6271 ab	8.42 b	0.3406 b
Bradyrhizobium sp. CNPSo 1083	26.9 b	0.4859 c	9.81 b	0.2855 c
Bradyrhizobium sp. CNPSo 1448	19.8 c	0.5666 bc	10.0 b	0.3529 b
Water Condition (WC)				
Control	24.7 a	0.6006 a	11.8 a	0.3393 b
PEG	21.2 b	0.4653 b	11.7 a	0.3737 a
ANOVA (*F probability*)				
SG	*ns*	**	*ns*	*Ns*
BNF	**	**	**	**
WC	**	**	*ns*	**
SG × BNF	*ns*	**	*ns*	**
SG × WC	*ns*	**	*ns*	*Ns*

BNF × WC	*ns*	*ns*	*ns*	*Ns*
SG × BNF × WC	*ns*	**	**	*
CV (%)	26.57	14.23	19.03	16.93

Soybean Genotypes: SG = BR14-4760 – progeny of PI 471938 x BRS 284; PI 471938 – drought tolerance; BRS 284 – drought sensitive.

Inoculation with BNF (BNF): Positive Control – (addition of N); inoculation at sowing with *Bradyrhizobium diazoefficiens* (strain SEMIA 5080/ CNPSo 06); *Bradyrhizobium elkanni* (strain CNPSo 014); *Bradyrhizobium diazoefficiens* (EUA) (strain CNPSo 056); *Bradyrhizobium* sp. (strain CNPSo 1083); *Bradyrhizobium sp.* (strain CNPSo 1448).

Water Condition: Control – Absence of PEG (Polyethylene glycol) 6000 solution; PEG- Application of PEG (Polyethylene glycol) 6000 solution.

Means (n = 4) followed by the same letter do not differ (*ns*) by SNK test at $*p \leq 0.05$ and $**p \leq 0.01$. For the variables CAT and APX the data is transformed by the root (x+k).

Table 5S. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and concentration of proline in nodules of soybean genotypes inoculated with different *Bradyrhizobium* strains under normal water supply or under osmotic stress induced by PEG 6000.

Factors	**SOD**	**CAT**	**APX**	**Proline**
Soybean Genotypes (SG)	mg protein^{-1}	µmoL min^{-1}	mg protein^{-1}	µmol g^{-1} FW
BR14-4760	21.7 a	0.4912 a	43.8 a	0.3709 a
PI 471938	30.1 a	0.5507 a	43.9 a	0.3880 a
BRS 284	28.9 a	0.5495 a	27.4 b	0.4418 a
Inoculation with BNF (BNF)				
B. diazoefficiens CNPSo 06	26.9 a	0.5895 a	42.7 a	0.4446 a
Bradyrhizobium sp. CNPSo 1448	26.9 a	0.4714 a	34.0 b	0.3559 b
Water Condition (WC)				
Control	30.9 a	0.4194 b	33.3 b	0.3276 b
PEG	22.8 b	0.6415 a	43.4 a	0.4728 a
ANOVA (*F probability*)				
SG	*ns*	*ns*	**	*ns*
BNF	*ns*	*ns*	*	**
WC	**	*	*	**
SG × BNF	*ns*	*ns*	*ns*	*

SG × WC	*ns*	*ns*	*	*ns*
BNF × WC	*ns*	*ns*	**	*ns*
SG × BNF × WC	*ns*	*ns*	*ns*	*ns*
CV (%)	16.90	21.32	14.97	27.67

Soybean Genotypes: SG = BR14-4760 – progeny of PI 471938 x BRS 284; PI 471938 – drought tolerance; BRS 284 – drought sensitive.
Inoculation with BNF (BNF): inoculation at sowing with *Bradyrhizobium diazoefficiens* (strain SEMIA 5080 = CNPSo 06) or *Bradyrhizobium* sp. (strain CNPSo 1448).
Water Condition: Control – Absence of PEG (Polyethylene glycol) 6000 solution; PEG- Application of PEG (Polyethylene glycol) 6000 solution.
Means (n = 3) followed by the same letter do not differ (ns) by SNK test at $*p \leq 0.05$ and $**p \leq 0.01$.

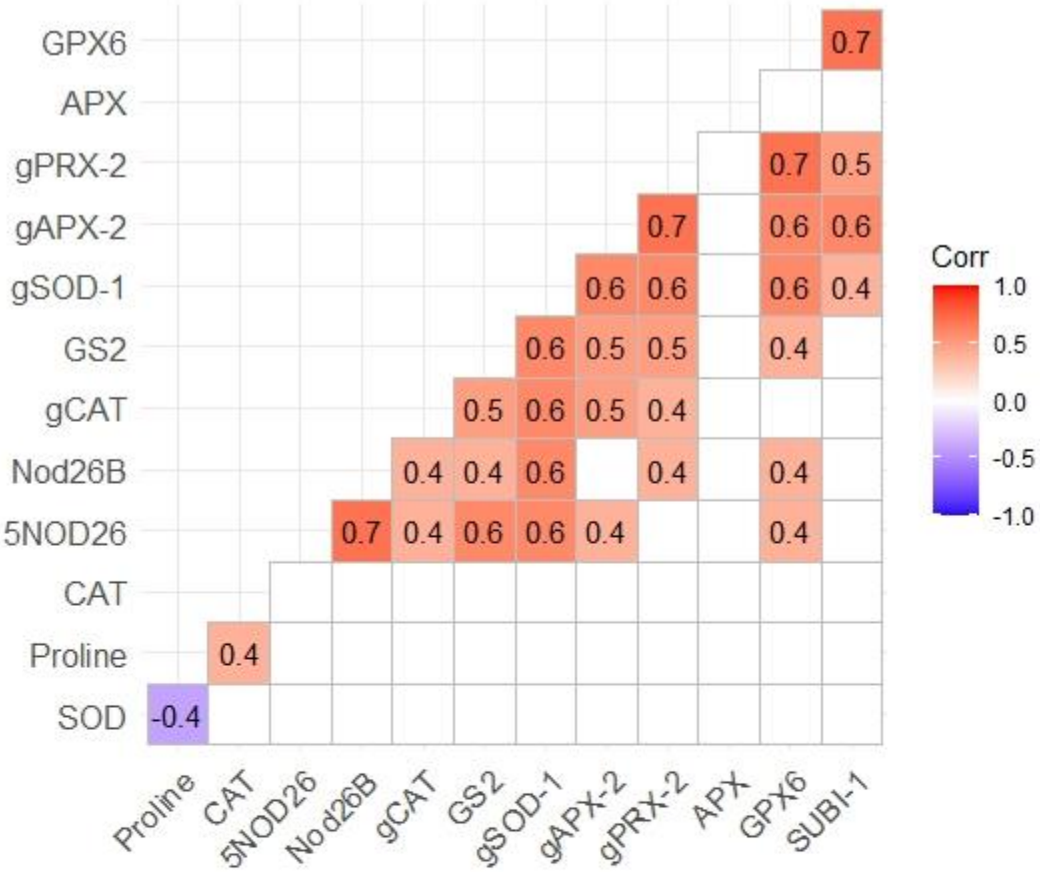

Fig. 1S. Heatmap of the Pearson's correlation coefficients (p ≤ 0.05) matrix between proline concentration, antioxidant enzyme, and nodule gene expression. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), proline, relative gene expression: catalase-peroxidase (gCAT), peroxiredoxin (gPRX), nodulin (Nod26B), glutamine synthetase (GS2), copper/zinc superoxide dismutase (gSOD-1), aquaporin (5NOD26), ascorbate peroxidase 2, cytosolic (gAPX-2), polyubiquitin (SUB-1), glutathione peroxidase (GPX6).

CAPÍTULO 3

REAPLICAÇÃO DE COBALTO, MOLIBDÊNIO e REINOCULAÇÃO de *Bradyrhizobium* sp., *Azospirillum brasilense* OU AMBOS EM SOJA APÓS OCORRÊNCIA DE SECA

REAPLICAÇÃO DE COBALTO, MOLIBDÊNIO e REINOCULAÇÃO de *Bradyrhizobium* sp., *Azospirillum brasilense* OU AMBOS EM SOJA APÓS OCORRÊNCIA DE SECA

RESUMO

O uso de inoculantes e cobalto-molibdênio na soja [*Glycine max* (L.) Merril] tem sido adotado há muitos anos na semeadura e/ou nas fases iniciais de desenvolvimento da cultura. No entanto, há evidências de que reaplicações suplementares tardias podem melhorar o desempenho da cultura, sobretudo após a ocorrência de estresses. A inoculação suplementar de *Bradyrhizobium* sp., *Azospirillum brasilense* ou a coinoculação de ambos, em combinação com a reaplicação de Cobalto e Molibdênio (CoMo) após ocorrência de seca foram avaliados em dois experimentos de campo na safra 2018/19 no sul do Brasil. Os tratamentos foram: controle não inoculado; *Bradyrhizobium* sp.; *Azospirillum brasilense*; coinoculação de ambos, em combinação com ou sem aplicação de CoMo aplicados via foliar, em fatorial 2 × 4 com 6 repetições. No primeiro experimento (com o genótipoBMX Ponta IPRO), o efeito do CoMo foi positivo para produtividade, número de vagens, número total de grãos, peso de grãos e peso de cem grãos. Para o segundo (com o genótipo DM 6563 IPRO), houve efeito positivo do CoMo para o número de grãos e massa de cem grãos, sem influenciar na produtividade, enquanto a coinoculação aumentou o teor de óleo no terço superior da planta e o número de grãos por planta, mas sem reflexo significativo na produtividade. A reaplicação de CoMo via foliar após ocorrência de estresse hídrico pode colaborar na recuperação da cultura da soja.

Palavras-chave: *Glycine max*, aplicação foliar, reinoculação tardia, micronutrientes, estresse hídrico.

Title

Reapplication of cobalt, molybdenum and reinoculation of *Bradyrhizobium* sp., *Azospirillum brasilense* or both in soybean after the occurrence of drought

Journal

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Abstract

The use of inoculants and cobalt-molybdenum in soybean [*Glycine max* (L.) Merril] has been adopted for many years at the sowing and/or at the early stages of crop development. However, there is evidence that late supplementary reapplications can improve crop performance, especially after the occurrence of stress. Supplemental inoculation of *Bradyrhizobium* sp., *Azospirillum brasilense* or co-inoculation of both, in combination with reapplication of cobalt and molybdenum (CoMo) after the occurrence of drought were evaluated in two field experiments in the 2018/19 crop season in southern Brazil. The treatments were: uninoculated control; *Bradyrhizobium* sp.; *Azospirillum brasilense*; co-inoculation of both, in combination with or without application of CoMo applied via foliar, in a 2 × 4 factorial with 6 replications. In the first experiment (with the genotype BMX Ponta IPRO), the effect of CoMo was positive for yield, number of pods, total number of grains, grain weight and weight of 100 grains. For the second experiment (with the genotype DM 6563 IPRO), there was a positive effect of CoMo for the number of grains and mass of 100 grains, without influencing yield, while co-inoculation increased oil concentration in the upper third of the plant and the number of grains per plant, but without significant impact on yield. The leaf reapplication of CoMo can collaborate in the recovery of soybean crop after drought stress.

Keywords: *Glycine max*, foliar application, secondary inoculation, micronutrients, water stress.

1. Introduction

Soybean (*Glycine max* L.) has great relevance worldwide as source of protein, oil, minerals, and carbohydrates. The soybean requirement for N reaches about 83 kg per metric ton of grains (Embrapa, 2020). However, biological nitrogen fixation (BNF) by inoculating elite strains of *Bradyrhizobium* can supply most part of the required N, and this process can be improved with co-inoculation with *Azospirillum brasilense*, resulting in high yields without the need for chemical N fertilizers (Rondina et al., 2020).

The BNF requires some micronutrients like cobalt (Co) and molybdenum (Mo) (Bárbaro et al., 2009; Galindo et al., 2017; Galindo et al., 2018; Comiran et al., 2020), which play major roles in this biological process. Cobalt is involved in the synthesis of leghemoglobin in nodules, whereas Mo takes part of the active sites of nitrogenase, nitrate reductase, and sulfide oxidase enzymes, and is involved in the electron transport during biochemical reactions (Sfredo and De Oliveira, 2010). Molybdenum also takes part in the nitrate reductase, involved in the processes of nitrate assimilation (Mengel and Kirkby, 2001).

Application of Co and Mo has provided a significant improvement in the efficiency of BNF in soybean. Galindo et al. (2017) found that the application of CoMo in soybean seeds and co-inoculation of *Bradyrhizobium* sp. and *A. brasilense* provided greater operational profit and profitability index, highlighting the need for co-inoculation and CoMo supplementation to improve yield and profitability.

However, the BNF is particularly vulnerable to drought, under which the decline in the nitrogenase activity is observed even before transpiration and photosynthesis rates (Bellaloui and Mengistu, 2015). Therefore, studies have been carried out to reduce impacts of drought on the production system. For this, agricultural management practices and plant breeding focusing on obtaining more effective symbionts under drought conditions have presented relevant results (Hungria and Vargas, 2000; Varshney et al., 2018; Freitas et al., 2022). The reapplication of CoMo combined with reinoculation with *Bradyrhizobium* and *A. brasilense* or both in co-inoculation can be a strategy to recover the effect of drought stress in soybean.

Therefore, the objective of this study was to assess the effect of reinoculation with *Bradyrhizobium* sp., *A. brasilense* or co-inoculation of both, combined with or without application of CoMo, in the recovery of soybeans after occurrence of drought.

2. Material and methods

2.1. Field experiments

Two experiments were set on commercial soybean areas in the municipality of Bela Vista do Paraíso, Paraná - Brazil in the 2018/19 crop season. The local climate is classified as Cfa (Humid, Subtropical, with hot summer), according to Köppen’s classification, and the soil is classified as Rhodic

Kandiudult (Soil Taxonomy, USDA). Meteorological data recorded during the experiment are shown in Figure 1.

Chemical attributes at 0-20 cm at the experimental sites were: Experiment I: 18 mg dm^{-3} P (resin); 38 g dm^{-3} OM; pH ($CaCl_2$) 5.2; K, Ca, Mg, Na, H + Al = 1.0; 10.2; 3.9; 0.06; 4.22 cmolc dm^{-3}, respectively; Cu, Fe, Mn, Zn = 24.2; 138; 691 and 11.2 mg dm^{-3}, respectively; and 78% of base saturation. Experiment II: 36 mg dm^{-3} P (resin); 13 g dm^{-3} OM; pH ($CaCl_2$) 5.6; K, Ca, Mg, Na, H + Al = 0.4; 4.1; 1.5; 0.06 and 3.8 cmolc dm^{-3}, respectively; Cu, Fe, Mn, Zn = 10; 220; 159 and 7,4 mg dm^{-3}, respectively; and 61% of base saturation.

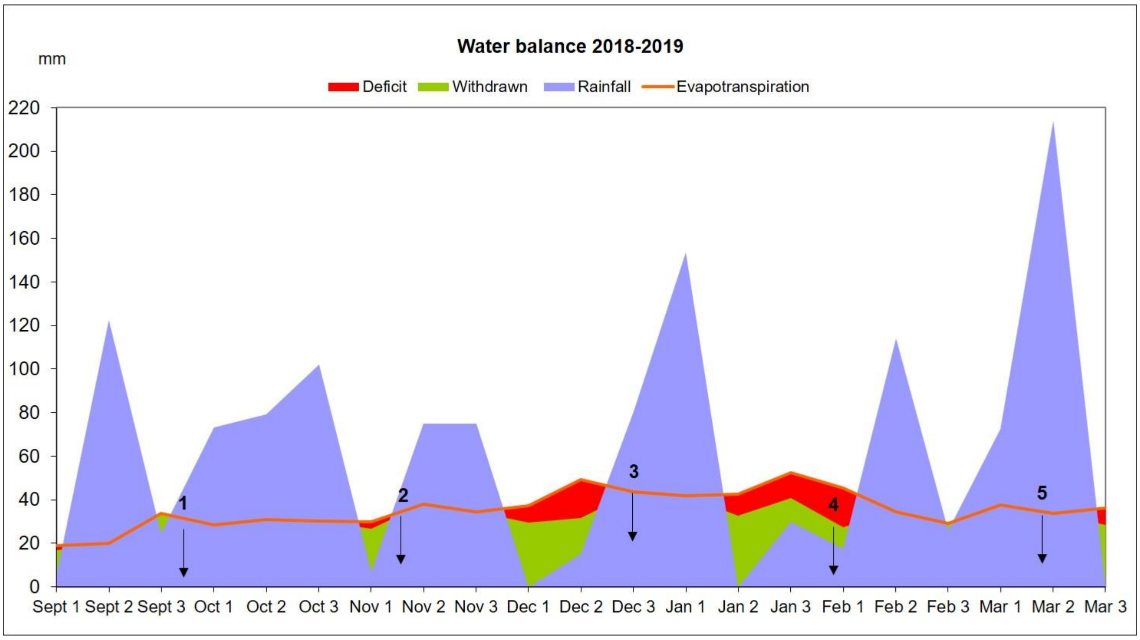

Figure 1. Ten-day climatological water balance during the soybean growth period during 2018/19 crop season. Bela Vista do Paraíso – PR, Brazil. Arrow 1 on September 25, 2018 and 2 on November 05, 2018 indicate the sowing dates for experiments I and II, respectively. Arrow 3 refers the application of treatments (inoculants and CoMo) carried out on December 18 and 22, 2018 for experiments I and II, respectively. Arrow 4 on January 29, 2019 and arrow 5 on March 8, 2019 indicate the harvest of experiments I and II, respectively.

2.2. Experimental plots and treatments

2.2.1 Sowing

Soybean was mechanically sown on September 25, 2018 with genotype BMS Ponta (early sowing, experiment I) and on November 2nd, 2018 with genotype DM 6563 IPRO (late sowing, experiment II).

In both experiments, fertilizers were applied at rates of 143 kg ha^{-1} of KCl and 269 kg ha^{-1} of single superphosphate at sowing. In experiment I, fertilizers were applied in furrow simultaneously to sowing; in experiment II fertilizers were applied 15 days before sowing. Soybean seeds were treated with the fungicide Maxin XL (0.04 L ha^{-1}), CoMo (Noduls Premium, 150 mL ha^{-1}), commercial inoculant

Gelfix® 5 containing *Bradyrhizobium elkanii* strains SEMIA 587 and SEMIA 5019 with minimal concentration of 5 × 10^9 CFU viable cells mL^{-1} and Biomax® Azum (*Azospirillum brasilense* Ab-v5 with minimal concentration of 3 × 10^8 CFU mL^{-1}.

2.2.2 Supplemental inoculation and application of CoMo after drought

The climate conditions were monitored during the experimental period to identify occurrence of water restriction to the crop growing in the field (Figure 1). The last significant rainfall occurred in the second and third week of November and became scarce until the second week of December leading plants to water stress to the wilting point during the hotter hours of the day. After about 80 mm rainfall between 16 and 17 December, treatments were applied on 18 and 22 December (Figure 1, arrow 3) to check their capacity to help plants to recover from stress. In homogeneous areas of the commercial crops, plots with 8 × 6 m were established, in six replications per treatment, distributed in six randomized blocks. Plants in experiment I were at the R5.3 developmental stage; in experiment II, plants were at the vegetative stage V5-6.

The treatments in both experiments were distributed in a split plot arrangement, in six replications, consisting of 4 levels of inoculation in the plots: 1. Control (no supplemental inoculation); 2. *Bradyrhizobium* sp. at 6 doses ha^{-1} (Biomax premium, 6.95 × 10^9 CFU mL^{-1}); 3. *A. brasilense* at 2 doses ha^{-1} (Azototal, 5. 64 × 10^8 CFU mL^{-1}); 4. Co-inoculation with both inoculants at the same doses. The subplots consisted of application or not of CoMo (Rizo Micro Dry – 150 mL ha^{-1}, 1,5% of Co, 30,0% of Mo). The applications were made on the plant canopy with pressurized CO_2 sprayer providing a flow rate of 200 L ha^{-1}, in which the biological products were applied before the CoMo in independent operations.

2.3. Evaluations

To evaluate yield components, five plants were collected from the central lines of each sub-plot at the maturation stage (R8). Plants in experiment I was harvested on January 29, 2019, and in experiment II on March 8, 2019. Yield components were assessed by third in plants - lower (L), medium (M), and upper (U) for a better detail on the effect of treatments on different parts of the plant. Were assessed number of grains; number of pods; total grains weight; 100 grains weight; and oil and protein concentrations in grains. Oil and protein were determined in a Thermo Scientific near-infrared spectroscopy (FT-NIR) equipment, model Antaris II, with an integrating sphere and reading in the range between 1100 and 2500 nanometers, as described by Tibola et al. (2018).

Yield was determined by harvesting plants at maturation stage (R8) in the four central lines in each experimental subplot. The harvests occurred on February 4, 2019, and March 12, 2019, for the first and second experiments, respectively. Grains were cleaned, weighed and the values adjusted to 13% moisture, after determining the humidity in a grain moisture tester (Gehaka Agri model G800, São Paulo, Brazil).

In the second experiment, in addition to the evaluation of yield components, the traits related to BNF were also assessed. Five plants were collected from central lines of each subplot at the reproductive stage R1. Shoots were separated from roots and dried at 65 ºC for 48 h to evaluate the shoot dry weight

(SDW). The nodules were picked from roots to determine the number of nodules (NN) and nodule dry weight (NDW). In addition, 10 leaves with the petiole of the third trifolium were collected and separated from petioles that were dried at 65 ºC for 48 h to determine N-ureides based on Vogels and Van Der Drift (1970).

2.4. Statistical analyses

Data were submitted to tests of normality and homogeneity of variances for each experiment, followed by the analysis of variance (ANOVA) with application of the F test ($P \leq 0.05$), and means comparison by Tukey's test ($P \leq 0.05$).

3. Results

The application of cobalt and molybdenum (CoMo) as strategy to help plant recovery after drought significantly affected yield components in both experiments. For experiment I, CoMo application increased yield and protein concentration in the lowest plant third (Table S1). In addition, number of pods, number of grains, total weight of grains, and 100 grains weight were also affected, depending on the plant third (Table S2). In experiment II there was significant difference for the yield components (Table S1); and number of grains, total grains weight, and 100 grains weight (Tables S1 and S2).

Regarding the supplemental inoculation, there was significant difference only in one of the plant third for the following traits: number of pods and grain weight in experiment I (Table S2). For both traits, in the upper portion of the plant, co-inoculation decreased values (-23%) compared with the control (Table 1).

For experiment II, inoculation affected oil concentration and 100-grains weight (Tables S1 and S2). Co-inoculation increased the oil concentration in the grains from the upper third by 4% over the control, in contrast to 100-grains weight in the lower third, in which inoculation, single or co-inoculated caused reduction over the control (Table 1).

Table 1. Effect of inoculation (Control, *Bradyrhizobium* sp. (Brady), *A. brasilense* (Azo) or co-inoculation (Brady+Azo) on number of pods, weight of grains, 100-grains weight; oil concentration in grains per third (lower – L, medium – M, and upper – U)). Bela Vista do Paraíso, PR, Brazil - 2018/2019 crop season.

Treatments	Number of pods			Weight of grains (g)			100-grains weight (g)			Oil %		
	L	M	U	L	M	U	L	M	U	L	M	U
	Experiment I – BMX Ponta IPRO											
Control	92 a	101 a	48 a	32.6 a	40.7 a	20.6 a	14.4 a	15.5 a	17.5 a	23.4 a	23.2 a	23.1 a
Brady	92 a	97 a	39 ab	36.3 a	42.9 a	17.7 ab	15.1 a	16.4 a	17.9 a	23.3 a	22.9 a	22.9 a
Azo	97 a	100 a	42 ab	34.4 a	39.9 a	18.1 ab	15.0 a	15.8 a	16.4 a	23.6 a	23.7 a	22.8 a
Brady+Azo	92 a	92 a	37 b	32.8 a	37.0 a	15.8 b	13.9 a	15.9 a	16.0 a	23.6 a	23.1 a	22.9 a
	Experiment II – DM 6563 IPRO											
Control	14 a	28 a	16 a	3.3 a	7.4 a	15.3 a	10.8 a	10.4 a	12.2 a	24.1 a	23.9 a	24.2 b
Brady	13 a	28 a	18 a	3.0 a	6.9 a	14.9 a	9.6 b	10.2 a	11.1 a	23.5 a	23.9 a	24.8 ab
Azo	12 a	28 a	17 a	3.0 a	6.7 a	14.5 a	10.0 b	10.4 a	11.4 a	23.3 a	24.0 a	24.7 ab
Brady+Azo	12 a	27 a	18 a	3.3 a	6.9 a	15.3 a	10.1 b	10.1 a	11.1 a	23.5 a	23.8 a	25.3 a

Means (n = 6) followed by the same letter in the column do not differ from one another (Tukey $p \leq 0.05$).

The supplemental application of CoMo increased crop yield by 11% and promoted positive effect for all yield components in at least one plant third in experiment I (Table 2). In contrast, supplemental CoMo application promoted a decrease by 194 kg ha^{-1} in grain yield in experiment II. The yield components were only slightly affected, with significant positive effect in the number of grains in the medium third, and weight of 100-grains in the upper third (Table 2).

Table 2. Yield, 100-grains weight, protein concentration, number of pods, total grains weight, and number of grains per third (lower – L, medium – M, and upper – U), according to application of Cobalt and Molybdenum (CoMo) in soybean after occurrence of drought. Bela Vista do Paraíso, PR, Brazil - 2018/2019 crop season.

Treat-ments	Yield (kg ha^{-1})	100-grains weight (g)			Protein %			Number of pods			Total grains weight (g)			Number of grains		
		L	M	U	L	M	U	L	M	U	L	M	U	L	M	U
	Experiment I – BMX Ponta IPRO															
CoMo	3806 a	14.5 a	16.4 a	17.6 a	34.4 b	36.3 a	37.9 a	94 a	105 a	46 a	35.4 a	44.6 a	20.5 a	245 a	271 a	117 a
Control	3388 b	14.6 a	15.4 b	16.3 a	35.3 a	36.4 a	38.2 a	92 a	90 b	37 b	32.7 a	35.7 b	15.6 b	223 b	232b	95 b
	Experiment II – DM 6563 IPRO															
CoMo	2645 b	10.2 a	10.2 a	11.8 a	34.1 a	34.4 a	34.0 a	13 a	28 a	18 a	3.2 a	7.4 a	5.1 a	31 a	72 a	43 a
Control	2838 a	10.1 a	10.3 a	11.1 b	34.7 a	34.7 a	34.0 a	13 a	27 a	17 a	3.1 a	6.6 b	4.7 a	30 a	64 b	42 a

Means (n = 6) followed by the same letter in the column do not differ each other (Tukey $p \leq 0.05$).

Plant nodulation (NN and NDW) in experiment II did not change with either supplemental inoculation or addition of CoMo. However, there was interaction between CoMo and inoculation on SDW and ureides, with inoculation effect for these traits depending on inoculation treatment (Table S3).

Inoculation with *Bradyrhizobium* sp. and co-inoculation with *A. brasilense* positively increased plant shoot biomass compared with the control. On the other hand, in the presence of CoMo there was no effect of inoculants. Considering each inoculation level in the interaction, the reapplication of CoMo in the non-inoculated plants increased shoot biomass by 12%. Conversely, in the inoculated plants, CoMo application reduced plant biomass by 12% when inoculated with *Bradyrhizobium* spp., 13% with *A. brasilense* and 23% in the coinoculation (Fig. 2a). Ureide concentration increased by 38% and 23% in petioles over non-inoculated plants when re-inoculated with *Bradyrhizobium* sp. or co-inoculated, respectively, only with CoMo re-application. There was no response to inoculation without CoMo re-application (Fig. 2b).

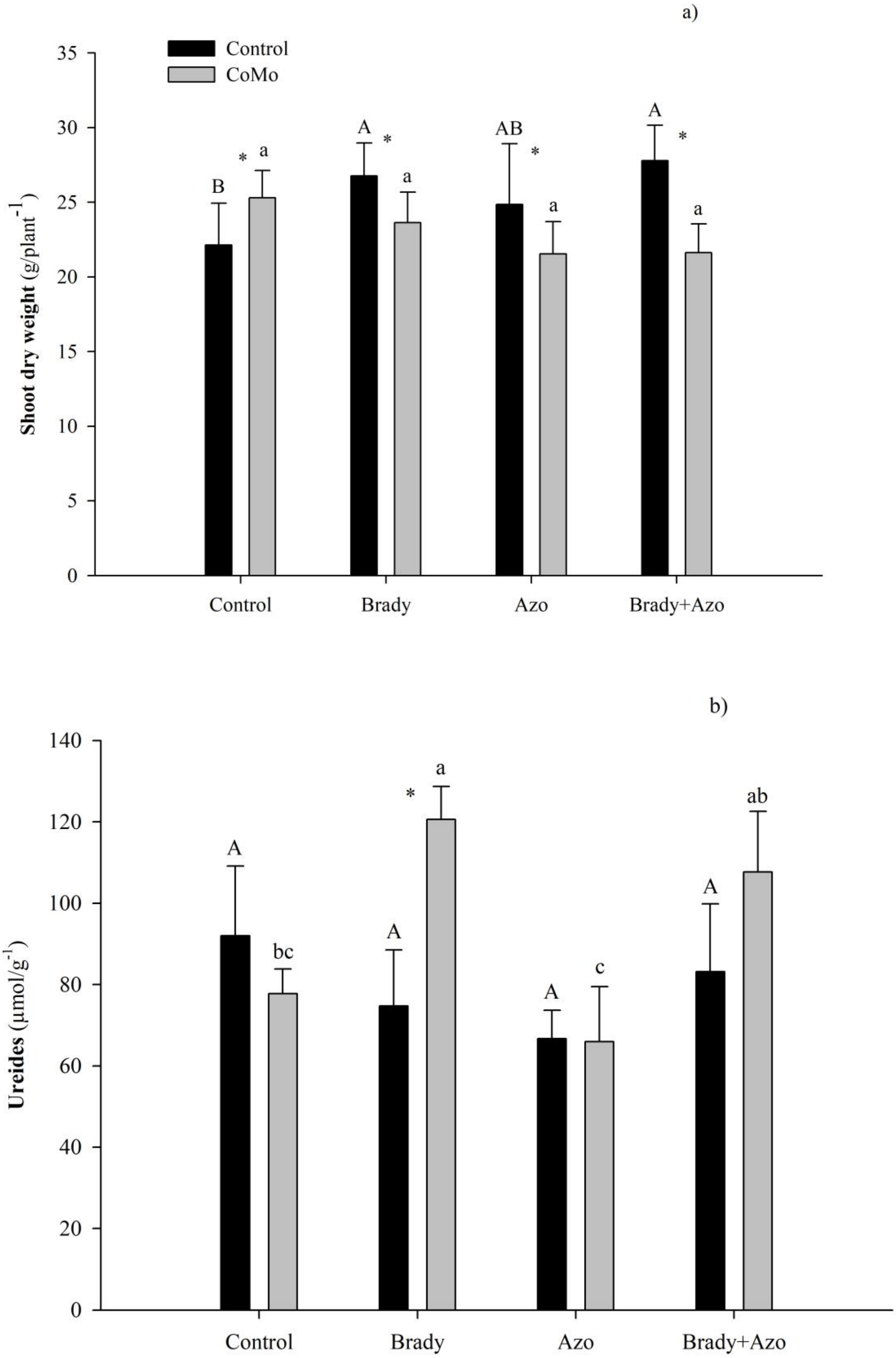

Figure. 2 Shoot dry weight (a), and ureides (b) in soybean genotype DM 6563 IPRO under interaction with inoculants (Control, *Bradyrhizobium* sp. (Brady), *A. brasilense* (Azo) or co-inoculation (Brady+Azo), in combination with or without re-application of Cobalt and Molybdenum (CoMo). Bela

Vista do Paraíso, PR, Brazil - 2018/2019 crop season. Means followed by the same letters do not differ from one another (Tukey, $p \leq 0.05$); Lowercase letters compare among inoculants within CoMo re-application; uppercase compare inoculants within absence of CoMo (Control). * Significant difference between CoMo (with/without) within each inoculation treatment (Tukey $p \leq 0.05$). Vertical bars represent the standard deviation (n = 6).

4. Discussion

Occurrence of drought during critical stages of plant development have caused yield losses and strategies to relieve the negative effect of drought on crops are welcome (Freitas et al., 2022). For soybean, the BNF process is particularly affected by drought and strategies to help the plant to recover the activity after stress can help to relieve yield losses or even improve the grain quality.

The co-inoculation after occurrence of drought increased oil concentration in grains from the upper third of the plant in experiment II. The upper portion of the plant usually presented equal or lower oil concentration in grains than the lower thirds among 10 (Sales et al., 2013) and 15 (Sales et al., 2016) soybean genotypes. Oil concentration in grains generally correlates inversely with protein concentrations (Marega-Filho et al., 2001), especially under stressing conditions during podfill, in which oil concentrations decreases while protein concentration increases (Rose, 1988; Mertz-Henning et al., 2018). As plants in experiment II were in an earlier developmental stage when inoculants were reapplied after drought, there was more time for plant recovery, as can be seen from plant biomass and concentration in ureides. However, a second wave of intense drought and high temperatures by the end of growing seasons seems to have caused more stress in the plants that had better recovered from the first wave, what may explain greater losses in yield and increase of oil concentration in the plants that received CoMo and co-inoculation. Protein concentration decreased in the lower third grains due to CoMo application in experiment I, although seed treatment with CoMo usually increases protein concentrations in grains under normal water supply (Meschede et al. 2004). However, supplemental CoMo application or inoculation after drought had only minor effects on protein concentration in grains in both experiments.

Supplemental inoculation with *Bradyrhizobium* sp. and co-inoculation increased shoot dry weight in experiment II compared with the control, in agreement with previous reports on beneficial results of inoculation and co-inoculation on soybean shoot biomass at sowing (Hungria et al. 2015; Rondina et al. 2020). Despite some improvements in ureides and plant biomass in experiment II, supplemental inoculation did not provide significant effect on grain yield in both experiments. Supplemental late inoculation under optimal climate conditions showed to be promising to increase soybean yield ranging between 2 to 7%, based on a second wave of nodulation (Moretti et al., 2018). Regular inoculation at sowing is a well-disseminated agronomic practice among Brazilian farmers providing by 8% yield increase (Hungria and Mendes, 2015) and may reach 16% with co-inoculation (Hungria et al., 2013). Yield components increase like number of pods and weight of 100-grains due to inoculation at sowing have also been observed (Dourado-Neto et al., 2012; Galindo et al. 2018). However these traits were not affected by supplemental inoculation after drought as assessed in experiment II.

Differently from Moretti et al. (2018), under irrigated conditions, the current trial was under rainfed conditions and as can be seen, the occurrence of rain was not regular, even after the worst period of drought (Figure 1) and may have impaired plant response to inoculation.

As observed in experiment II supplemental inoculation or supplemental application of CoMo did not affect NN or NDW, in opposition to previous findings (Bárbaro et al., 2009; Gewehr, 2019), but applications were made on seeds at sowing or at early vegetative stages. Comiran et al. (2020) also observed no significant effect of leaf application of CoMo on soybean nodulation. In addition to plant developmental stages, several factors may interact with the effect of Co and Mo on the crop response, like soil organic matter contents, soil fertility and acidity, and the soil capacity to supply required amount of these micronutrients (Meschede et al., 2004). Even the concentration of Mo in the seed cotyledons (Campo et al., 2009) and plant genotype (Libório et al., 2020) may affect the plant response to external application of Mo.

Regular application of CoMo in seed treatment or at early vegetative states, associated with co-inoculation have led to significant increases in soybean yield by 990 kg ha^{-1} of grains more than the non-treated and non-inoculated control (Galindo et al. 2017). In experiment I, supplemental application of CoMo increased yield in 418 kg ha^{-1} (11%), emphasizing the use of supplemental application of CoMo after drought to help the plant to recover from stress. Application of CoMo at sowing or early stages improves grain yield (Sfredo and Oliveira, 2010; Hungria et al., 2007; Dourado Neto et al., 2012; Galindo et al. 2017) in a range from 200 to 1.020 kg ha^{-1}. Yield components also increased due to supplemental application of CoMo after drought. Application of CoMo via seed and/or leaf in the vegetative stage usually promotes increase yield of soybeans, as a result of increase in the number of pods, grains per pod, weight thousand seeds (Dourado Neto et al., 2012). However, this is the first report on yield components improvement due to supplemental application of CoMo after occurrence of drought as strategy to recover from the stress.

In contrast to positive effects on yield in experiment I, the application of CoMo decreased plant yield in experiment II by 193 kg ha^{-1} (-6,8%) compared with the control. As hypothesis, plants that received supplemental application of CoMo may have started a recovery process, as observed for ureides in plants inoculated with *Bradyrhizobium* sp., but a second wave of drought (Figure 1) may have been more impacting to these plants, leading to losses. In fact, in plants re-inoculated with *Bradyrhizobium* sp. and, to a lesser extent, co-inoculated, expressed an increase in the concentration of ureides, which is an indicative of BNF improvement (King and Purcell, 2005; Herridge and Peoples, 1990; Rosso et al., 2021). Despite this apparent recovery, yield had a general decrease with reapplication of CoMo.

Conclusions

Our research revealed that reapplication of CoMo and inoculants after severe drought may alleviate the stress effect on some plant traits related to BNF and yield components, but the effect on grain yield depend on other factors like plant developmental stage and re-incidence of stressing conditions.

New experiments should be carried out with the objective of confirming the effect of CoMo in the recovery of water deficit, being another alternative to alleviate the negative impacts of the drought.

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Supplementary Information

Table S1. Analysis of variance for yield, 100-grain weight, and oil and protein concentrations per plant third (lower – L, medium – M, and upper – U), according to inoculation (*Bradyrhizobium* sp., *A. brasilense* or co-inoculation), application of Cobalt and Molybdenum (CoMo), and the interaction inoculation × CoMo. Bela Vista do Paraíso, PR, Brazil - 2018/2019 crop season.

Variation Source	Mean Square (MS)							
	Yield (kg ha^{-1})	100-grain weight (g)	Oil %			Protein %		
			L	M	U	L	M	U
	Experiment I – BMX Ponta IPRO							
Inoculation	66916.74^{ns}	0.25^{ns}	0.32^{ns}	1.45^{ns}	0.22^{ns}	1.05^{ns}	3.00^{ns}	0.17^{ns}
Residue (A)	217573.03	0.29	0.35	0.54	1.05	0.40	0.85	1.38
CoMo	2102125.52*	0.05^{ns}	1.01^{ns}	0.51^{ns}	0.00^{ns}	9.92*	0.07^{ns}	1.36^{ns}
Inoc*CoMo	100925.19^{ns}	0.01^{ns}	1.25^{ns}	0.46^{ns}	2.01^{ns}	2.05^{ns}	0.40^{ns}	2.31^{ns}
Residue (B)	196325.37	0.44	0.56	0.74	0.96	0.89	0.99	0.86
CV (%) A	9.17	2.29	1.79	2.25	3.16	1.28	1.79	2.18
CV (%) B	12.32	3.99	3.20	3.71	4.26	2.71	2.74	2.44
Mean	3597	16.7	23.5	23.2	22.9	34.9	36.4	38.1
	Experiment II – DM 6563 IPRO							
Inoculation	68484.97^{ns}	0.05^{ns}	1.57^{ns}	0.04^{ns}	2.35*	1.64^{ns}	0.69^{ns}	0.59^{ns}
Residue (A)	54502.95	0.26	1.51	2.34	0.51	1.07	1.60	0.82
CoMo	449694.08*	0.09^{ns}	2.05^{ns}	0.27^{ns}	0.62^{ns}	4.25^{ns}	0.64^{ns}	0.90^{ns}
Inoc*CoMo	62368.97^{ns}	0.17^{ns}	0.09^{ns}	2.66^{ns}	0.98^{ns}	0.52^{ns}	1.63^{ns}	0.61^{ns}
Residue (B)	88648.65	0.09	1.20	1.04	1.20	0.99	1.18	0.95
CV (%) A	6.02	3.05	3.68	4.53	2.04	2.13	2.59	1.88
CV (%) B	10.86	2.64	4.64	4.28	4.43	2.90	3.15	2.85
Mean	2742	11.8	23.6	23.9	24.8	34.4	34.5	34.2

^{ns} not significant; * significant at 5% probability by the F test. CV = Coefficient of variation.

Table S2 Analysis of variance for number of pods, number of grains, total grains weight, 100-grain weight per third (lower – L, medium – M, and upper – U) in soybean plants, according to inoculation (*Bradyrhizobium* sp., *A. brasilense* or co-inoculation), with or without application of Cobalt and Molybdenum (CoMo), and the interaction inoculation × CoMo. Bela Vista do Paraíso, PR, Brazil - 2018/2019 crop season.

Variation Source	Mean Square (MS)											
	Number of pods			Number of grains			Total weight grains (g)			100-grains weight (g)		
	L	M	U	L	M	U	L	M	U	L	M	U
	Experiment I – BMX Ponta IPRO											
Inoculation	15.44^{ns}	42.48^{ns}	47.91*	124.4^{ns}	498.7^{ns}	201.07^{ns}	6.88^{ns}	14.32^{ns}	9.65*	0.69^{ns}	0.33^{ns}	1.90^{ns}
Residue (A)	32.02	78.43	12.08	359.30	533.48	84.78	3.91	14.52	2.35	0.42	0.38	1.54
CoMo	9.60^{ns}	604.84*	207.20*	1131*	3728.8*	1118.02*	17.59^{ns}	188.4*	58.85*	0.024^{ns}	2.47*	3.49^{ns}
Inoc*CoMo	52.20^{ns}	33.55^{ns}	10.57^{ns}	56.28^{ns}	190.3^{ns}	55.38^{ns}	2.77^{ns}	7.77^{ns}	3.37^{ns}	0.56^{ns}	0.07^{ns}	1.26^{ns}
Residue (B)	68.44	50.07	7.40	175.92	442.62	92.11	5.89	12.07	2.79	0.45	0.27	1.03
CV (%) A	9.61	14.35	13.27	12.80	14.52	13.70	9.19	15.01	13.43	7.00	6.12	11.57
CV (%) B	19.86	16.21	14.69	12.67	18.71	20.19	15.95	19.36	20.71	10.29	7.33	13.37
Mean	18.62	19.52	8.28	46.81	50.27	21.26	6.80	8.03	3.61	2.92	3.18	3.39
	Experiment II – DM 6563 IPRO											
Inoculation	12.80^{ns}	6.75^{ns}	10.39^{ns}	30.30^{ns}	56.80^{ns}	110.19^{ns}	0.31^{ns}	0.93^{ns}	0.31^{ns}	0.58*	0.06^{ns}	0.66^{ns}
Residue (A)	7.28	22.63	15.92	40.30	141.39	69.24	0.39	1.32	0.94	0.06	0.35	0.22
CoMo	0.75^{ns}	21.33^{ns}	27.00^{ns}	5.33^{ns}	736.33*	15.19^{ns}	0.05^{ns}	6.52*	1.63^{ns}	0.01^{ns}	0.07^{ns}	1.04*
Inoc*CoMo	3.58^{ns}	27.22^{ns}	8.28^{ns}	53.00^{ns}	166.39^{ns}	107.35^{ns}	0.58^{ns}	1.93^{ns}	0.96^{ns}	0.02^{ns}	0.25^{ns}	0.19^{ns}
Residue (B)	6.52	22.00	10.26	39.13	101.82	56.56	0.53	1.29	0.72	0.14	0.25	0.236
CV (%) A	14.64	12.14	16.28	14.58	12.28	13.74	14.12	11.61	14.04	3.87	9.06	6.46
CV (%) B	19.59	16.93	18.48	20.32	14.74	17.57	23.22	16.25	17.37	8.25	10.82	9.51
Mean	13.04	27.71	17.33	30.79	68.46	42.81	3.13	6.99	4.87	2.03	2.05	2.29

^{ns} not significant;* significant at 5% significance by the F test. CV = Coefficient of variation.

Table S3. Analysis of variance for shoot dry weight (SDW), number of nodules (NN), nodule dry weight (NDW) and ureides in soybean plants in experiment II, according to inoculation (*Bradyrhizobium* sp., *A. brasilense* or co-inoculation), with or without application of Cobalt and Molybdenum (CoMo), and the interaction inoculation × CoMo. Bela Vista do Paraíso, PR, Brazil - 2018/2019 crop season.

Treatments	SDW (g)	NN	NDW (mg)	Ureides (µmol/g)
Inoculation	9.87 ns	741.41 ns	8013.50ns	1634*
Residue (A)	10.79	825.94	10134.03	-
CoMo	66.57*	31.69 ns	432.00 ns	1533*
Inoc*CoMo	46.50*	126.69 ns	4806.05 ns	1424*
Residue (B)	6.20	337.14	3288.19	282
CV (%) A	9.59	25.81	21.18	-
CV (%) B	10.29	23.32	17.06	19.49
Mean	24.21	78.73	336.00	86.09

^{ns} not significant; * significant at 5% significance by the F test. CV = Coefficient of variation.

CONCLUSÃO GERAL

Frente a previsões sobre alterações climáticas e os prejuízos causados pela seca na cultura da soja, destaca-se neste trabalho a relevância da manutenção da fixação biológica de nitrogênio para o melhor enfretamento da soja mediante ao estresse hídrico. No entanto, não há uma solução única para favorecer a tolerância à seca, mas um conjunto de estratégias que devem ser empregadas para diminuir as oscilações de produtividade frente a condições hídricas não favoráveis. As pesquisas para tolerância à seca devem ir além da planta hospedeira, incluindo também sua interação com insumos empregados no sistema de produção, químicos ou biológicos, além do manejo adequado do solo no sistema de produção.

As diferenças genotípicas em soja com características contrastantes em relação à FBN sob seca e a combinação de diferentes estirpes de *Bradyrhizobium* desencadeiam respostas específicas sob estresse osmótico, podendo influenciar na tolerância ao estresse. A combinação do genótipo de soja BR14-4760 com a estirpe de *Bradyrhizobium diazoefficiens* CNPSo 06 (= SEMIA 5080) destacou-se como a melhor interação para o enfrentamento do estresse hídrico, provavelmente pelo fato deste genótipo tolerar por mais tempo uma redução de água no solo, com manutenção da taxa fotossintética e redução da temperatura foliar, indicando um processo de regulação de defesa contra o estresse. Por sua vez, isolados mais tolerantes e eficientes em FBN também podem resultar em interações mais resilientes. Apesar dos indicativos promissores em condições controladas, são necessárias avaliações em condições reais para verificar o desempenho desse genótipo em interação com a estirpe em destaque e outros insumos biológicos, como a coinoculação com *Azospirillum*, adição de metabólitos secundários e aplicação suplementar de CoMo que podem favorecer ainda mais a resposta da planta sob seca.

Por fim, verificou-se, em condições de campo, que a reaplicação de CoMo após a ocorrência de seca promoveu efeitos nas plantas mais evidentes do que a reinoculação ou coinoculação com *Bradyrhizobium* sp. e *Azospirillum brasilense*, melhorando alguns atributos da planta, mas o efeito na produtividade varia com a intensidade do estresse e com a fase do desenvolvimento da planta.