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PAULA CEREZINI

**VARIAÇÕES GENOTÍPICAS E USO DE MICRORGANISMOS
E METABÓLITOS MICROBIANOS COMO ESTRATÉGIAS
PARA MITIGAR OS EFEITOS DA RESTRIÇÃO HÍDRICA À
FIXAÇÃO BIOLÓGICA DE NITROGÊNIO EM SOJA**

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Orientador: Dr. Marco Antonio Nogueira.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”.

(Madre Teresa de Calcutá)

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RESUMO

A fixação biológica de nitrogênio (FBN) é indispensável para a viabilidade econômica e ambiental da cultura da soja, mas estresses ambientais, como a seca, prejudicam a FBN e a cultura. O objetivo geral dessa tese foi identificar variações genotípicas em soja quanto a tolerância à seca, com base em componentes de produção, atividade da FBN e da taxa transpiratória sob condições de seca, além de avaliar estratégias agrônômicas para sustentação de altas taxas da FBN na cultura da soja sob restrição hídrica. O primeiro artigo apresenta o resultado da avaliação de seis genótipos de soja do programa de melhoramento genético da Embrapa Soja para tolerância à seca, quanto ao metabolismo do N e produção de grãos, em experimento em condições controladas. Os genótipos BRB-27, R02-1325 e BRB-26, que têm um parental tolerante à seca, apresentaram maior produção de grãos após o período de restrição hídrica, sugerindo maior capacidade de recuperação após reidratação. O segundo artigo documenta a capacidade de recuperar a transpiração e a FBN (pela técnica da redução do acetileno) em dez genótipos de soja provenientes do programa de melhoramento genético da Universidade Estadual da Carolina do Norte para tolerância à seca, expostos à restrição hídrica severa e reidratação, em condições controladas. A recuperação da transpiração e da atividade de FBN variou entre três e quatro dias, considerada rápida se comparada a genótipos sensíveis à seca. Variações genotípicas em relação à capacidade de recuperação foram identificadas, mas as diferenças foram sutis e provavelmente não resultariam em maior desempenho da cultura no campo. No terceiro artigo avaliou-se o efeito da coinoculação de *Bradyrhizobium* com *Azospirillum* e da adição de metabólitos secundários microbianos (MSM) na FBN e produtividade da soja sob diferentes condições climáticas em quatro ensaios a campo. A coinoculação aumentou a nodulação da soja nos estágios iniciais e resultou em maior crescimento vegetativo e acúmulo de N na parte aérea, especialmente em condições de seca. A adição de MSM atenuou o efeito da seca sobre a nodulação e em um experimento aumentou a produção de grãos em 15% e 7% em relação ao tratamento que recebeu N mineral e ao tratamento que recebeu somente inoculação com *Bradyrhizobium*, respectivamente. A seleção e utilização de genótipos de soja tolerantes à seca, juntamente com práticas sustentáveis que contribuam para a manutenção da FBN, são potenciais ferramentas para atenuar efeitos negativos da restrição hídrica na cultura da soja.

Palavras-chave: Fotossíntese. Glutamina sintetase. Nitrogenase. Tolerância à seca. Ureídeos.

CEREZINI, Paula. **Genotypic variations and use of microorganisms and microbial metabolites as strategies to mitigate the effects of water restriction on biological nitrogen fixation in soybean**, 2016. 80p. Thesis (PhD in Agronomy), Universidade Estadual de Londrina, Londrina-PR, 2016.

ABSTRACT

Biological nitrogen fixation (BNF) is essential for the economic and environmental viability of the soybean crop, but environmental stresses such as drought impair the FBN and the crop. In this thesis, the general aim was to identify genotypic variations in soybean as drought tolerance, based on yield components, activity of BNF and transpiration rates under drought conditions, and also evaluate agronomic strategies for sustaining of high rates of BNF in soybean under water restriction. The first paper presents the results of the assessment of six soybean genotypes from the program of genetic breeding for drought tolerance of the Embrapa Soybean, regarding to N metabolism and grain yield, in a trial under controlled conditions, to characterize soybean genotypes with BNF tolerant to drought. The genotypes BRB-27, R02-1325 and BRB-26, which have a drought-tolerant parental, presented higher grain yield capacity after exposure to drought, suggesting better capacity to recover from drought after rewetting. The second paper documented the plant capacity to recover the transpiration and BNF (by acetylene reduction activity) in 10 soybean genotypes from the program of genetic breeding for drought tolerance of the North Carolina State University, after exposure to severe water stress followed by rehydration, under controlled conditions. The recovery of transpiration and BNF varied between three to four days, considered fast if compared with drought-sensitive genotypes. Genotypic variation in the recovery rate was identified, but not expected to have a large impact on plant performance in the field. In the third paper, we evaluated the effect of co-inoculation of *Bradyrhizobium* with *Azospirillum* and addition of microbial secondary metabolites (MSM) on BNF and soybean yield under different climatic conditions in four field trials. Co-inoculation increased nodulation of soybeans at the early stages of crop development and resulted in increased plant growth and accumulation of N in the shoot, especially under dry conditions. The addition of MSM attenuated the effect of drought on nodulation and in one trial increased the grain yield by 15% and 7% in relation to the N-fertilizer and sole inoculation with *Bradyrhizobium*, respectively. Selection and use of soybean genotypes drought tolerant, along with sustainable practices that contribute to keep the BNF, are potential tools for attenuation of negative effects of drought on soybean.

Keywords: Photosynthesis. Glutamine synthetase. Nitrogenase. Drought tolerance. Ureides.

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1 INTRODUÇÃO

A viabilidade econômica da cultura da soja (*Glycine max* L. Merrill) no Brasil está estreitamente relacionada à fixação biológica do nitrogênio (FBN). Esse processo é realizado por bactérias do gênero *Bradyrhizobium*, que são capazes de fixar o N₂ atmosférico em nódulos radiculares e fornecer a maior parte do N requerido pela cultura, o que dispensa o uso de fertilizantes químicos nitrogenados, os quais possuem altos custos econômicos e ambientais.

Entre os fatores abióticos mais limitantes à produtividade da cultura da soja, destaca-se a seca, que afeta a planta fisiológica e nutricionalmente, com prejuízos ao processo de FBN, que é especialmente vulnerável à privação de água no solo. Com isso, o suprimento de N à cultura é diretamente afetado, o que causa limitações à produtividade e, conseqüentemente, prejuízos econômicos. Os fatores envolvidos na redução/inibição da FBN sob seca ainda não são bem compreendidos, mas alguns estudos têm associado a sensibilidade da FBN à seca com um, ou a combinação de mecanismos: (1) limitação na difusão de oxigênio para o nódulo, (2) escassez de substratos de carbono ao bacteroide e (3) regulação pelo metabolismo do nitrogênio, com acúmulo de compostos nitrogenados, como os ureídeos, nas folhas e nódulos (SERRAJ; SINCLAIR; PURCELL, 1999; GÁLVEZ; GONZÁLEZ; ARRESE-IGOR, 2005; CERZINI et al., 2014).

Contudo, existem variações entre genótipos de soja quanto à capacidade de manter a FBN, como também seus processos fisiológicos, durante a restrição hídrica moderada. As linhagens R01-581F e R01-416F, progênies da cultivar 'Jackson', identificada por apresentar FBN tolerante à seca, foram selecionadas, testadas e demonstraram potencial de manter a FBN em condições de seca (CHEN et al., 2007a). Já os genótipos PI 416937 e PI 471938 foram selecionados por expressar fenótipo de murchamento lento (*slow-wilting*), quando submetidos à seca em condições de campo, e estão sendo usados em programas de melhoramento para o desenvolvimento de cultivares de soja tolerantes à seca (DEVI; SINCLAIR, 2013). A capacidade de recuperação dos processos fisiológicos da planta e da atividade da FBN é outra característica esperada em genótipos de soja melhor adaptados a períodos de escassez de água, visto que esse atributo pode refletir em maior estabilidade na produção de grãos da cultura após um veranico.

Estratégias que auxiliem no estabelecimento e manutenção do processo de FBN, tais como a coinoculação e uso de metabólitos secundários microbianos (MSM) visando ao estímulo da nodulação, também têm sido exploradas, a fim de aumentar a eficiência da FBN em leguminosas, especialmente em regiões tropicais. A coinoculação de *Bradyrhizobium* com bactérias promotoras do crescimento de plantas (BPCPs), como *Azospirillum*, tem se mostrado benéfica, pois aumenta o crescimento vegetal, estimula a nodulação precoce e melhora a FBN em várias leguminosas, promovendo incrementos na produtividade (HUNGRIA; NOGUEIRA; ARAUJO, 2013). Os oligossacarídeos lipoquitínicos (LCOs) são MSM que atuam como moléculas sinalizadoras da simbiose; os LCOs purificados têm ação benéfica em raízes de plantas leguminosas por favorecer o estabelecimento dos nódulos e aumentar a eficiência da FBN (CULLIMORE; RANJEVA; BONO, 2001; MARKS et al., 2013). Com o estabelecimento mais precoce da simbiose e o aumento da eficiência da FBN, o uso dessas técnicas pode resultar em aumento da tolerância da planta à seca e da sustentabilidade do sistema de produção de soja.

Há crescente interesse em pesquisas direcionadas a estratégias inovadoras que contribuam para maximizar a FBN, a absorção de outros nutrientes e a promoção do crescimento vegetal. Além disso, estudos para identificação de genótipos de soja com características relacionadas à tolerância à seca e capacidade de recuperação de suas funções metabólicas após o período adverso é uma ferramenta importante para uso em programas de melhoramento genético visando maior estabilidade da produtividade da cultura.

O objetivo desse trabalho foi (1) caracterizar respostas de genótipos de soja, quanto ao metabolismo do N e produção de grãos após ocorrência de deficiência hídrica; (2) documentar a capacidade de recuperação da transpiração e da FBN em soja após período de restrição hídrica severa e reidratação; (3) avaliar o efeito da coinoculação de *Bradyrhizobium* com *Azospirillum* e adição de MSM, na FBN e produtividade da cultura da soja sob diferentes condições climáticas.

2 REVISÃO DE LITERATURA

2.1 CULTURA DA SOJA

2.1.1 Produção e Importância Econômica

A soja é uma das leguminosas de maior relevância econômica no cenário agrícola mundial, com produção de 315 milhões de toneladas de grãos na safra 2015/16 (CONAB, 2016). Isso se deve à grande aplicabilidade de seus grãos, que apresentam elevados teores de proteína e óleo, especialmente na nutrição animal e humana, sendo utilizados na agroindústria, indústria química e alimentícia, além da produção de biocombustível (DALL'AGNOL et al., 2007; HARTMAN; WEST; HERMAN, 2011; HIRAKURI; LAZZAROTO, 2014; HUNGRIA; MENDES, 2015). A produção brasileira, na safra 2015/16, atingiu 96,5 milhões de toneladas de grãos, com 33.251,9 mil hectares plantados, o que representa cerca de 57% da área cultivada com grãos do país (CONAB, 2016). Atualmente, os Estados Unidos são os maiores produtores mundiais de soja, produzindo cerca de 10% mais que o Brasil, o segundo produtor (CONAB, 2016). No entanto, perspectivas indicam que até 2023 a safra de soja brasileira deverá ultrapassar a norte americana (FIESP, 2015).

A cultura da soja deu início à ascensão agrícola brasileira (DALL'AGNOL et al. 2007; TOLLEFSON, 2010) e possibilitou a consolidação do Brasil como um dos principais fornecedores mundiais dessa oleaginosa. Uma vez que a produção da soja está entre as atividades agrícolas que mais cresceram nas últimas décadas, o seu comércio se tornou uma das principais fontes de divisas do agronegócio brasileiro (FIESP, 2015; CONAB, 2016).

O sucesso do agronegócio brasileiro da soja pode ser atribuído a diversos fatores, dentre os quais: investimentos em pesquisa científica para o desenvolvimento de cultivares adaptadas ao solo e às condições climáticas do país (ALVES; BODDEY; URQUIAGA, 2003), desenvolvimento e estruturação de um sólido mercado internacional relacionado ao comércio de produtos do complexo agroindustrial da soja, a consolidação da oleaginosa como importante fonte de proteína vegetal e a geração e oferta de tecnologias ao setor produtivo (HIRAKURI; LAZZAROTO, 2014). Outro fator muito importante para a consolidação da soja no país foi a seleção de bactérias fixadoras de nitrogênio eficazes nas condições

brasileiras, bem como o desenvolvimento de inoculantes comerciais (HUNGRIA; CAMPO; NOGUEIRA, 2012), o que tornou a produção de soja no Brasil independente do uso de fertilizantes químicos nitrogenados (FAGAN et al., 2007; HUNGRIA; CAMPO; MENDES, 2007).

Projeções indicam que a população mundial aumentará dos atuais 7,3 bilhões para 8,5 bilhões de habitantes em 2030, e mais de 9 bilhões em 2050 (UNITED NATIONS, 2015). Isso significa um aumento na demanda mundial por alimento, água e energia de 35%, 40% e 50%, respectivamente (IPEA, 2015; HARTMAN; WEST; HERMAN, 2011), que exigirá um incremento na produção agrícola e exploração das terras cultiváveis. Para isso, esforços em pesquisas com a cultura da soja são fundamentais para o desenvolvimento de tecnologias que visem dar sustentabilidade e, ao mesmo tempo, reduzir custos do sistema de produção frente a incertezas decorrentes das mudanças climáticas globais. Nesse cenário, a utilização de recursos genéticos por meio da seleção e desenvolvimento de genótipos tolerantes aos estresses abióticos é uma ferramenta importante, visando soluções necessárias para amenizar ou combater a contínua pressão de fatores adversos à produção (SADOK; SINCLAIR, 2011; FOYER et al., 2016).

1.1.2. Fixação Biológica de Nitrogênio

Com exceção da água, o nitrogênio (N) é o componente que mais limita o crescimento e desenvolvimento de plantas. Esse nutriente tem papel estrutural, genético e metabólico, e participa do crescimento e desenvolvimento das plantas, com influência direta no rendimento das culturas (CHRISTOPHE et al., 2011; OHYAMA et al., 2013).

Apesar de o N ser o elemento mais abundante na atmosfera terrestre, as plantas e outros organismos eucariotos são incapazes de utilizá-lo. Isto ocorre porque o N atmosférico está na forma de N_2 , que possui alta estabilidade, devido à tripla ligação covalente entre os átomos de N, uma das mais fortes de que se tem conhecimento na natureza (FAGAN et al., 2007; HOFFMAN et al., 2014). No entanto, como resultado de milhões de anos de evolução, algumas espécies pertencentes à família Leguminosae (=Fabaceae), que compreende três subfamílias (Caesalpinioideae, Mimosoideae e Papilionoideae), desenvolveram a capacidade de estabelecer simbiose com bactérias da classe Proteobacteria, que são capazes de

fixar N_2 e disponibilizá-lo às plantas (SPRENT, 2007; WERNER et al., 2014; BARAL; SILVA; IZAGUIRRE-MAYORAL, 2016).

A fixação biológica do nitrogênio (FBN) consiste na transformação do N_2 em formas assimiláveis pelas plantas, e é realizada por alguns microrganismos procariotos, denominados diazotróficos, que, no caso da soja, fornecem a maior parte do N que a planta necessita, desde que respeitados os procedimentos para uma nodulação rápida e eficiente (HUNGRIA et al., 2006). Em troca, a planta fornece fotoassimilados, essenciais para a manutenção do metabolismo bacteriano e da FBN (FAGAN et al., 2007; HUNGRIA; CAMPO; MENDES, 2007). O processo de FBN ocorre em temperatura e pressão ambientes, resultando em uma fonte de N de baixo custo econômico e, ao mesmo tempo, propicia altos rendimentos à cultura da soja, com impacto ambiental reduzido (HUNGRIA; CAMPO; MENDES, 2007). A economia para o Brasil com a não utilização dos fertilizantes nitrogenados e aplicação dessa tecnologia na produção de soja é estimada em US\$ 15 bilhões anualmente (HUNGRIA; MENDES, 2015).

Devido à importância desse processo biológico para a agricultura brasileira, a FBN foi incluída como uma das metas do Programa ABC, no Plano Setorial de Mitigação e de Adaptação às Mudanças Climáticas visando à Consolidação de uma Economia de Baixa Emissão de Carbono na Agricultura, como parte do compromisso internacional assumido pelo Brasil, em 2009, de reduzir suas emissões de Gases de Efeito Estufa (GEE) entre 36,1% e 38,9%, até 2020. Nesse programa assume-se o compromisso de expansão de uso da FBN em 5,5 milhões de hectares no país (MAPA, 2011), como alternativa sustentável ao uso de nitrogênio mineral.

O processo de FBN envolve uma série de etapas que se iniciam com a atração da bactéria por sinais moleculares, em geral flavonoides, excretados pelas raízes das plantas e culminam na fixação do N_2 . Na infecção e colonização das raízes ocorrem mudanças fisiológicas, bioquímicas e morfológicas, tanto na célula hospedeira, quanto do microrganismo (FAGAN et al., 2007; BARAL; SILVA; IZAGUIRRE-MAYORAL, 2016). O estabelecimento da simbiose entre leguminosas e microrganismos fixadores de N_2 , popularmente chamados de rizóbios, não é obrigatória; no entanto, sob condições limitadas de N no solo, os simbiontes procuram-se uns aos outros, por meio de trocas de sinais moleculares, tendo como resultado a formação de um novo órgão na raiz da planta, o nódulo (TAIZ; ZEIGER,

2013; RIBEIRO et al., 2014). No nódulo, a bactéria é diferenciada em bacteroide e a partir da formação da simbiose, diversas enzimas são sintetizadas, como o complexo enzimático nitrogenase, e proteínas, como a leghemoglobina (FAGAN et al., 2007; RIBEIRO et al., 2014).

O complexo nitrogenase é formado por duas unidades proteicas, a Ferro-proteína (Fe-proteína) e a Molibdênio-Ferro-proteína (MoFe-proteína), que são capazes, juntamente com a ferridoxina, de transportar elétrons que são necessários para a redução do N_2 a NH_3 (DANCE, 2013). A enzima nitrogenase é sensível à exposição ao oxigênio (O_2), mesmo em baixas concentrações, sendo irreversivelmente inativada. A leghemoglobina regula o transporte de O_2 para as células fixadoras de N_2 nos nódulos, de modo que a sua concentração seja mantida em nível não deletério à nitrogenase, mas que permita a respiração do bacteroide visando a obtenção de energia e poder redutor empregados na FBN. Os nódulos, quando ativos, apresentam coloração rosácea intensa em seu interior, indicando a atividade da leghemoglobina (FAGAN et al., 2007; HUNGRIA; CAMPO; MENDES, 2007; SINCLAIR; VADEZ, 2012).

No nódulo, o N atmosférico é reduzido pela nitrogenase a amônia (NH_3), que, em contato com o substrato aquoso do citoplasma dos bacteroides, é transformada em íon amônio (NH_4^+); este é liberado por difusão para o citosol da célula infectada (OHYAMA et al., 2013; BARAL; SILVA; IZAGUIRRE-MAYORAL, 2016). O acúmulo de NH_4^+ é tóxico ao bacteroide e à célula hospedeira, por isso deve ser convertido a formas orgânicas, tais como ureídeos, aminoácidos e amidas, para posterior translocação e incorporação no metabolismo do N das plantas. As reações de assimilação são normalmente intermediadas pelas enzimas glutamina sintetase (GS) e glutamato sintase (GOGAT). Assim, a atividade destas enzimas é um dos parâmetros que indicam eficiência na assimilação do N e, conseqüentemente, na atividade da FBN (FAGAN et al., 2007; CHRISTOPHE et al., 2011).

Em geral, leguminosas de clima temperado transportam N na forma de amidas, mas, no caso da soja, 90% do N fixado é transportado na forma de ureídeos (KING; PURCELL, 2005; SINCLAIR et al., 2007; CHRISTOPHE et al., 2011). As principais formas de ureídeos presentes nas plantas são a alantoína, sintetizada nos peroxissomos, a partir do ácido úrico, e o ácido alantoico, sintetizado no retículo endoplasmático, a partir da alantoína (KING; PURCELL, 2005). Esses compostos

nitrogenados são translocados, via xilema, dos nódulos até a parte aérea, onde são rapidamente metabolizados (KING; PURCELL, 2005). A relação C:N de 1:1 da alantoína confere uma grande vantagem para os ureídeos como molécula de transporte, fornecendo o N para as plantas com custos reduzidos de C, enquanto que para amino-compostos (asparagina e glutamina) a relação C:N é de 2:1 (ALCÂNTARA; REIZ, 2008; BARAL; SILVA; IZAGUIRRE-MAYORAL, 2016).

Os benefícios à planta promovidos pelo microssimbionte, pela disponibilização do N, são acompanhados de um alto custo energético para a planta hospedeira, visto que os nódulos demandam carbono como fonte de energia ao microssimbionte e para fornecer esqueletos de carbono à assimilação do amônio produzido (SCHUBERT, 2002). No entanto, os custos gerados pela FBN são compensados pelo aumento da taxa fotossintética, que pode aumentar em até 28% estimulada pela simbiose por causa do forte dreno por fotoassimilados (KASCHUK et al., 2009). Como o N é essencial à síntese da Rubisco, principal enzima envolvida na fixação do CO₂ e na síntese de clorofilas, a FBN, ao aumentar a concentração de N foliar, aumenta também a eficiência fotossintética (KASCHUK et al., 2010). Com o estímulo da fotossíntese pela simbiose, o período para o crescimento da planta e enchimento de grãos pode aumentar, adiando a degradação de proteínas foliares e da clorofila, com retardamento da senescência da planta, o que pode resultar em maiores rendimentos para a cultura (ONO et al., 2001; KASCHUK et al., 2010).

No entanto, como a maioria dos processos fisiológicos das plantas, a atividade da FBN pode sofrer interferências sob condições adversas, com destaque para os fatores ambientais, tais como: disponibilidade hídrica (seca ou alagamento), temperatura e salinidade elevadas, que são agentes determinantes para o estabelecimento e sucesso da relação simbiótica (SPRENT, 1972; CHRISTOPHE et al., 2011; SINCLAIR; VADEZ, 2012; HUNGRIA; KASCHUK, 2013).

2.2 ALTERAÇÕES CLIMÁTICAS

Nos últimos anos, alterações no clima têm sido observadas em todo o mundo. Isso inclui aumento na temperatura da Terra e diminuição das geleiras do Ártico, irregularidade da precipitação pluvial, alteração da salinidade dos oceanos, dos padrões de ventos e a ocorrência de eventos climáticos extremos, tais como secas, fortes precipitações, ondas de calor e intensidade de ciclones tropicais,

levando a alterações no equilíbrio energético do sistema climático (IPCC, 2011; IPCC, 2014; SHANKER et al., 2014).

O efeito estufa é um fenômeno natural, que ocorre na atmosfera e possibilita as condições térmicas necessárias para a vida na Terra. Os principais GEE são: dióxido de carbono (CO_2), metano (CH_4), ozônio (O_3) e óxido nitroso (N_2O) (IPCC, 1995). Esses gases, junto com o vapor d'água, absorvem parte dos raios infravermelhos refletidos pela superfície terrestre, impedindo que essa energia se dissipe para o espaço. Essa interação permite que a temperatura média da atmosfera seja favorável à vida. Caso não houvesse esses gases na atmosfera, a temperatura média da Terra seria, aproximadamente, 30 °C menor, o que inviabilizaria as atuais formas de vida (CERRI et al., 2007).

As atividades antrópicas, como o crescente consumo de combustíveis fósseis, a derrubada e queima de florestas, mudanças no uso da terra e os processos industriais têm contribuído para o aumento excessivo dos GEE na atmosfera. O acúmulo contínuo desses gases acarreta maior retenção de radiação infravermelha emitida pela Terra, com conseqüente aumento da temperatura do ar e decorrentes eventos extremos (IPCC, 2007; IPCC, 2011). Nas últimas décadas, as mudanças climáticas globais têm despertado preocupações da comunidade científica (IPCC, 2011; WANG et al., 2012). A problemática destas alterações levou a OMM (Organização Meteorológica Mundial) e o PNUMA (Programa das Nações Unidas para o Meio Ambiente) a criarem o IPCC (Painel Intergovernamental sobre Mudanças Climáticas) em 1988, que é considerado como órgão consultor de todas as nações para avaliar as questões relacionadas às mudanças no clima (IPCC, 2007).

No mais recente relatório sobre as alterações climáticas (IPCC, 2014) foi divulgado que a influência humana no sistema climático é clara, e entre os impactos causados pelas mudanças do clima é previsto o aumento da temperatura média do planeta, com aumento do nível do mar decorrente do derretimento de geleiras e, com isso, milhões de pessoas sob risco de inundações, escassez de água potável, impacto sobre a segurança alimentar e extinção de espécies.

Em relação à agricultura, há risco de perda de meios de subsistência rurais e renda devido ao insuficiente acesso a água potável e de irrigação, com redução da produtividade agrícola, em particular para os agricultores de regiões semi-áridas (IPCC, 2014). Segundo o IPCC (2007), dentre os cenários de mudanças

climáticas, foi prevista a redução de até 20% das chuvas em áreas subtropicais até 2100, com agravamento das secas. De fato, nos últimos anos tem havido maior irregularidade das chuvas durante o ciclo das culturas, sendo a ocorrência de períodos secos, denominados veranicos, cada vez mais frequentes, trazendo seguidos prejuízos ao agronegócio (CHAVES; OLIVEIRA, 2004; ASSAD et al., 2013).

2.3 RESTRIÇÃO HÍDRICA

2.3.1 Prejuízos à Produção de Soja

A relação entre agricultura e o clima é bastante complexa, pois fatores ambientais afetam tanto o crescimento e desenvolvimento das plantas, como também as etapas do cultivo, incluindo preparo do solo, época de semeadura, colheita, transporte e armazenagem do produto. Como todas as práticas agrícolas, o cultivo de soja sofre influência de diversos fatores e, entre eles, a seca é um dos principais agentes abióticos de redução de produtividade do grão (EMBRAPA, 2010; SHANKER et al., 2014).

A distribuição da vegetação sobre a superfície da Terra é controlada principalmente pela disponibilidade de água, que constitui cerca de 80-90% da massa fresca das plantas. O déficit hídrico pode ser definido como todo o conteúdo de água de um tecido ou célula que está abaixo do nível exibido no estado de hidratação plena (TAIZ; ZEIGER, 2013). A resposta das culturas frente à restrição hídrica é considerada uma síndrome, ou seja, um grupo de respostas simultâneas (LAWLOR, 2002). A escassez de água afeta todas as etapas do ciclo da cultura da soja, desde a germinação e floração, até o enchimento, desenvolvimento e qualidade dos grãos (MANAVALAN et al., 2009). No Brasil, a irregularidades nos padrões de chuva nos últimos dez anos ocasionaram prejuízo aproximado de 27 bilhões de dólares na produção de soja em dois dos mais importantes estados produtores da oleaginosa, Rio Grande do Sul e Paraná, onde as plantações deixaram de produzir mais de 55 milhões de toneladas de grãos nesse período (EMBRAPA, 2014).

Na safra de soja brasileira de 2011/2012, a estiagem prolongada causou diminuição na produção da oleaginosa em 11,8%, representando 8,93

milhões de toneladas de grãos a menos, em relação à produção da safra anterior (2010/2011), que tinha sido de 75,32 milhões de toneladas (CONAB, 2012). Na safra 2013/2014, a seca também trouxe prejuízos em algumas regiões produtoras de soja no Brasil, como no norte do Paraná, onde o tempo seco e quente foi prejudicial às lavouras, sobretudo as semeadas mais tardiamente (CONAB, 2014; CERZINI et al., 2016). A produtividade média nacional alcançada na safra 2015/16 foi de 2.870 kg ha⁻¹, 4,3% menor que safra 2014/15, fato atribuído às adversidades climáticas, o que representou decréscimo de 0,8%, mesmo com aumento de 3,6% da área plantada (CONAB, 2016).

A necessidade da soja por água varia de 450 mm a 800 mm ao longo do ciclo. A semente de soja necessita absorver pelo menos 50% de sua massa em água para assegurar a germinação. Nessa fase, o conteúdo de água no solo não deve exceder a 85% da capacidade de retenção máxima e nem ser inferior a 50%. A exigência por água aumenta com o desenvolvimento da planta, atingindo o máximo na floração/enchimento de grãos (7 a 8 mm dia⁻¹), decrescendo após esse período (EMBRAPA, 2010). Em trabalhos realizados em Londrina - PR, ao longo de 15 safras, avaliando-se diversas cultivares de soja em diferentes condições de disponibilidade hídrica, verificou-se que os maiores rendimentos de grãos foram obtidos com 650 a 700 mm de água bem distribuídos ao longo do ciclo da cultura (FARIAS; NEPOMUCENO; NEUMAIER, 2007).

Como parte do processo evolutivo, as plantas desenvolveram mecanismos para suportar condições adversas, como a seca. Em resposta ao estresse, as plantas ativam uma série de mecanismos de defesa; essas reações são mediadas por vários genes que levam à produção de proteínas que atuam sinergicamente em vias metabólicas visando ao aumento da tolerância/resistência à restrição hídrica (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000). As respostas fisiológicas e bioquímicas decorrentes incluem: alongação do sistema radicular, diminuição da área foliar, acúmulo de açúcares na parte aérea, entre outros (FARIAS; NEPOMUCENO; NEUMAIER, 2007; TAIZ; ZEIGER, 2013).

2.3.2 Prejuízos à Fixação Biológica de Nitrogênio

O efeito da seca na FBN em soja tem sido amplamente documentado por ser considerado o principal fator ambiental que afeta a eficiência

simbiótica, com consequências negativas ao rendimento da cultura (ZAHARAN, 1999; LADRERA et al., 2007; SINCLAIR et al., 2007; CERZINI et al., 2016). A FBN em soja é caracterizada por ser bastante vulnerável à restrição hídrica, considerado o primeiro processo fisiológico a ser prejudicado, antes mesmo da transpiração e da fotossíntese (KING; PURCELL, 2005; ARRESE-IGOR et al., 2011), talvez porque os processos de trocas gasosas sejam parte do metabolismo primário das plantas que influenciam diretamente na performance do hospedeiro e conseqüentemente na do simbiote.

A restrição hídrica compromete a formação da simbiose e a sobrevivência do microsimbiote, com redução no número, massa e metabolismo dos nódulos, e redução da atividade da nitrogenase (SERRAJ; SINCLAIR; PURCELL, 1999; STREETER, 2003; CHRISTOPHE et al., 2011). Isso pode ser atribuído ao elevado custo de C necessário à biossíntese e manutenção dos nódulos pela planta, que compete por nutrientes com outras partes em crescimento, tais como raízes, folhas, caule e sementes (VOISIN et al., 2003). Três principais fatores são sugeridos como responsáveis pela redução/inibição da FBN sob seca: (1) limitação de oxigênio no interior dos nódulos, (2) a escassez de substratos de carbono e (3) a regulação retroinibitória pelo metabolismo do nitrogênio (SERRAJ; SINCLAIR; PURCELL, 1999; KING; PURCELL, 2001; GÁLVEZ; GONZÁLEZ; ARRESE-IGOR, 2005; LADRERA et al., 2007; CERZINI et al., 2014; NASR et al., 2014).

Em situação de restrição hídrica a permeabilidade dos nódulos aos gases diminui e limita o fornecimento de O₂ para a respiração do bacteroide, visto que a camada de células corticais dos nódulos diminui a difusão de gases durante um estresse fisiológico (HUNGRIA; VARGAS, 2000; MARINO; GONZÁLEZ; ARRESE-IGOR, 2006; CHRISTOPHE et al., 2011). Além disso, em condições severas de seca, o conteúdo de leghemoglobina nos nódulos diminui (MANAVALAN et al., 2009), comprometendo ainda mais o fornecimento de O₂ ao microsimbiote; como consequência, a atividade da nitrogenase é reduzida (CHRISTOPHE et al., 2011), em parte por causa de danos oxidativos. A disponibilidade hídrica também é essencial para a importação e assimilação de fotoassimilados nos nódulos, bem como exportação e metabolismo de compostos nitrogenados na parte aérea. Quando esses processos são afetados sob restrição hídrica ou elevadas temperaturas, há interferência no metabolismo do C e do N que atua na regulação

da FBN nos nódulos (LADRERA et al., 2007; ARRESE-IGOR et al., 2011; HUNGRIA; KASCHUK, 2013; CERZINI et al., 2014).

A limitação de substratos de carbono ao microssimbionte é atribuível ao decréscimo na atividade da sacarose sintase (SS), principal enzima responsável pela hidrólise da sacarose nos nódulos (GONZÁLEZ et al., 1995). Assim, ocorre acúmulo de carbono nos nódulos na forma de sacarose, que não pode ser usada pelo bacteróide para suprir seu requerimento de energia. Em contrapartida, esgotam-se as fontes de carbono como ácidos dicarboxílicos, principalmente malato e succinato, necessários para a obtenção de energia e poder redutor pelo bacteróide, o que diminui a atividade da nitrogenase (GÁLVEZ; GONZÁLEZ; ARRESE-IGOR, 2005; LADRERA et al., 2007; MARINO et al., 2007; ALCANTARA; REIS, 2008; ARRESE-IGOR et al., 2011).

O metabolismo do N também interfere na regulação da FBN em condições de seca, pelo *feedback* resultante do acúmulo de compostos nitrogenados na parte aérea e nos nódulos. O acúmulo se dá tanto pela carência de água para o transporte desses compostos, quanto pela inibição de enzimas envolvidas no metabolismo do N (HUNGRIA; VARGAS, 2000; VADEZ; SINCLAIR, 2002; CERZINI et al., 2014). Várias moléculas orgânicas tiveram sua participação sugerida nesse mecanismo, como ureídos e aminoácidos livres (VADEZ; SINCLAIR; SERRAJ, 2000; KING; PURCELL, 2005; SINCLAIR et al., 2007; CERZINI et al., 2014), sendo que plantas que transportam N na forma de amidas são menos sensíveis ao déficit hídrico moderado do que aquelas que transportam ureídos, como a soja (SERRAJ; SINCLAIR; PURCELL, 1999; SINCLAIR et al., 2007).

Vários estudos têm relacionado a sensibilidade da FBN à seca com acúmulo de ureídos em diferentes partes da planta (SERRAJ; SINCLAIR; PURCELL, 1999; DEVI; SINCLAIR, 2013; CERZINI et al., 2014; MARQUEZ-GARCIA et al., 2015). Em soja, os ureídos são os principais produtos da FBN exportados dos nódulos para a parte aérea (SPRENT, 1980). A vantagem dos ureídos no transporte do N fixado está não somente na economia de carbono, mas também no menor gasto de ATP para sua síntese, 1-4 ATP, em comparação com 5-7 ATP gastos na produção de glutamato e asparagina (MINCHIN et al., 1981). No entanto, os ureídos possuem baixa solubilidade, o que implica na necessidade de maior volume de água para o seu transporte, 2,6 vezes mais do que o necessário para transportar

quantidades equivalentes de N na forma de asparagina (SPRENT, 1980; GOI; NEVES, 1982).

Por outro lado, a baixa solubilidade da alantoína permite o uso desse composto como armazenamento temporário (SPRENT, 1980). Plantas perenes de clima temperado (*Acer*, por exemplo) podem estocar ureídos durante períodos de baixas temperaturas e pouca água disponível e transportá-los na primavera. Isso permite alta translocação de N para a produção de novas folhas, com o uso mínimo de carboidratos, que nesta fase estariam escassos (MOTHES, 1961). Uma situação similar pode ocorrer nos nódulos e pecíolos de soja sob restrição hídrica. Além disso, estudos têm observado acúmulo de ureídos na parte aérea de soja sob seca (KING; PURCELL, 2005; DEVI; SINCLAIR, 2013; CEREZINI et al., 2014), devido ao decréscimo do catabolismo de ureídos nas folhas. Isso pode acelerar o processo de senescência das plantas e ainda afetar negativamente a atividade da nitrogenase (SERRAJ; SINCLAIR; PURCELL, 1999; KING; PURCELL, 2005).

Estirpes de rizóbios, espécies de plantas e mesmo variações genotípicas dentro da mesma espécie apresentam variação quanto à tolerância à seca (SINCLAIR et al., 2007; DEVI; SINCLAIR, 2013; CEREZINI et al., 2014; CEREZINI et al., em preparação). Além disso, a associação entre plantas e bactéria promotoras de crescimento de plantas (BPCP) pode ser altamente benéfica, especialmente em condições de baixa fertilidade do solo e baixa disponibilidade hídrica (HUNGRIA; NOGUEIRA, ARAUJO, 2013; CEREZINI et al., 2016). Assim, estudos e aplicação dessas estratégias podem trazer efeitos positivos para o cultivo da soja sob seca.

2.4 ESTRATÉGIAS PARA AUMENTAR A TOLERÂNCIA DA SOJA À SECA

A deficiência hídrica influencia muitos processos fisiológicos indiretamente; plantas sob estresse hídrico têm afetados o fechamento estomático com consequência negativa às trocas gasosas, o que prejudica a transpiração, a fotossíntese, o crescimento, as atividades enzimáticas, o metabolismo do N e C, entre outros processos metabólicos (FARIAS; NEPOMUCENO; NEUMAIER, 2007; CHRISTOPHE et al., 2011; SINCLAIR et al., 2007), refletindo negativamente no rendimento da cultura. Entretanto, as plantas quando submetidas à restrição hídrica,

devido à capacidade de percepção do estresse, apresentam mecanismos morfofisiológicos de aclimação, a fim de reduzir a perda de água para o ambiente (CATTIVELLI et al., 2008; TAIZ; ZEIGER, 2013). Essas alterações ocorrem desde as raízes até a parte aérea das plantas e incluem mecanismos como o fechamento estomático e redução da transpiração, com conseqüente redução da perda de água (FLEXAS et al., 2006; TAIZ; ZEIGER, 2013); limitação no crescimento da parte aérea a fim de poupar energia e alongação do sistema radicular para melhor explorar a água disponível no solo (CATTIVELLI et al., 2008; LOPES et al., 2011); ajuste osmótico pelo acúmulo de solutos que propiciam redução do potencial osmótico e manutenção da pressão de turgor celular (CATTIVELLI et al., 2008); entre outros.

Alterações morfofisiológicas induzidas pela ocorrência da situação adversa, como qualquer outro mecanismo, possuem base molecular. A percepção, por receptores específicos, da condição de seca pela planta induz uma cascata de eventos moleculares que levam à indução da expressão de várias categorias de genes (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000). Estudos têm identificado alguns genes que respondem à desidratação, dentre eles uma família de genes que codificam fatores de transcrição denominados DREB (“Dehydration Responsive Element Binding protein”) foi identificada (SHINOZAKI; YAMAGUSHI-SHINOZAKI, 2000; YAMAGUSHI-SHINOZAKI; SHINOZAKI, 2005). Esses fatores de transcrição participam da ativação de outros genes que levam à proteção das estruturas celulares durante a desidratação celular (YAMAGUSHI-SHINOZAKI; SHINOZAKI, 2005). Com o intuito de induzir tolerância à seca em várias espécies vegetais, a introdução por engenharia genética do fator de transcrição DREB tem sido feita em plantas como tabaco, arroz, trigo, soja e algodão, (YAMAGUSHI-SHINOZAKI; SHINOZAKI, 2005; CHEN et al., 2007b).

2.4.1 Variabilidade Genotípica da Planta

Variações genotípicas entre cultivares de soja quanto à sensibilidade da FBN à seca têm sido documentadas em vários estudos em casa de vegetação e a campo (VADEZ; SINCLAIR, 2002; SINCLAIR et al., 2007; ARRESE-IGOR et al., 2011; CEREZINI et al., 2014; DEVI et al., 2014). A cultivar ‘Jackson’ foi selecionada, em 1987, entre 28 genótipos, por apresentar características de FBN tolerante à seca

(SALL; SINCLAIR, 1991). Serraj e Sinclair (1997) confirmaram a superioridade de 'Jackson' quanto à tolerância à seca, em comparação com 18 cultivares de soja.

A vantagem fisiológica deste genótipo é atribuída à sua habilidade em minimizar o acúmulo de ureídeos na planta sob seca, pois os metaboliza rapidamente nas folhas. Essa capacidade foi associada ao maior acúmulo Mn na parte aérea (SERRAJ; SINCLAIR, 1997; PURCELL; KING; BALL, 2000; VADEZ; SINCLAIR, 2002), uma vez que a enzima amidohidrolase, responsável pela degradação de ureídeos nas folhas, requer Mn como cofator (VADEZ; SINCLAIR; SERRAJ, 2000). Entretanto, estudos posteriores mostram que o catabolismo dos ureídeos na parte aérea independe do Mn, pois outras vias enzimáticas podem ser utilizadas (SINCLAIR; VADEZ; CHENU, 2003; CHARLSON; KORTH; PURCELL, 2009; ALAMILO et al., 2010).

A cultivar 'KS4895' (PI 595081) tem alto potencial produtivo, mas possui FBN sensível à seca (PURCELL; KING; BALL, 2000). 'Jackson' (PI 548657), apesar de tolerante à seca, possui menor potencial produtivo. Em 1993, realizou-se o cruzamento dessas cultivares com a obtenção de várias linhagens. Em 2001, os genótipos R01-581F e R01-416F foram selecionados entre 600 linhagens, e entre 2002 e 2006 demonstraram maior potencial de rendimento em condições de seca (CHEN et al., 2007a). Sinclair et al. (2007) estudaram os genótipos R01-581F e R01-416F em experimentos em casa de vegetação e a campo, sob restrição hídrica, e observaram maior acúmulo de N na parte aérea e atividade da FBN em relação à parental 'KS4895', além de maiores rendimentos de grãos em relação aos controles comerciais em condições de seca moderada.

Recentemente, dois genótipos de soja (PI 416937 e PI 471938) que expressam o fenótipo de murcha lenta (*slow-wilting*) sob restrição hídrica estão sendo usados em programas de melhoramento para o desenvolvimento de linhagens tolerantes à seca em diferentes países (SINCLAIR; ZWIENIECKI; HOLBROOK, 2008; DEVI; SINCLAIR, 2013; CERZINI; RIAR, SINCLAIR, 2016). No genótipo PI 416937 essa característica está associada à sua capacidade de reduzir as taxas transpiratórias com o aumento do déficit de pressão de vapor (DPV), resultando na conservação da água no solo (SINCLAIR; ZWIENIECKI; HOLBROOK, 2008). Já o genótipo PI 471938 não possui o mesmo ajuste fisiológico que a PI 416937, pois não foi observada nenhuma vantagem fisiológica quanto ao fechamento estomático antecipado, redução da transpiração com o aumento do

DPV, quando comparado com outras cultivares comerciais de soja. Sadok et al. (2012) não conseguiram identificar um mecanismo fisiológico para explicar o fenótipo de murchamento lento observado em PI 471938. Entretanto, este genótipo mantém alta atividade da FBN, baixo acúmulo de ureídos nas folhas e maior turgescência na planta sob deficiência hídrica, mas as investigações ainda são inconclusivas quanto aos mecanismos envolvidos (DEVI; SINCLAIR, 2013).

Além da capacidade de tolerar períodos de déficit hídrico, é importante no processo de seleção de genótipos de soja avaliar a habilidade de recuperação da planta após a ocorrência de seca moderada seguida de reidratação (ROSAS-ANDERSON et al., 2014; CEREZINI; RIAR; SINCLAIR, 2016). A capacidade da planta em recuperar suas atividades metabólicas após o estresse influencia diretamente no seu potencial produtivo (JAMES; LAWN; COOPER, 2008). Apesar de os efeitos da seca em soja ser bem documentado durante a ocorrência do estresse, poucos estudos têm investigado as respostas da planta após um período de restrição hídrica seguido de reidratação.

Variações genótípicas quanto à capacidade de recuperação dos processos fisiológicos após reidratação foram observadas em estudos recentes com soja e amendoim (JAMES; LAWN; COOPER, 2008; ROSAS-ANDERSON et al., 2014; CEREZINI; RIAR; SINCLAIR, 2016). Os mecanismos de tolerância à seca e recuperação são dependentes da manutenção do conteúdo de água nos tecidos, da economia de água ou uma integração de ambos (ROSAS-ANDERSON et al., 2014). Cerezini, Riar e Sinclair (2016) observaram diferenças entre parentais e progênies quanto à capacidade de recuperação da FBN e da transpiração da planta após restrição hídrica severa, visto que genótipos que apresentaram bom desempenho quanto à manutenção desses processos sob estresse não se destacaram quanto à recuperação dos processos fisiológicos após restabelecimento do fornecimento de água. Futuras pesquisas nessa área são necessárias para elucidar esses mecanismos e contribuir para a seleção de genótipos de soja tolerantes à seca.

2.4.2 Bactérias Promotoras de Crescimento de Plantas

As bactérias promotoras de crescimento de plantas (BPCP) são microrganismos de vida livre ou que vivem associados às raízes e proporcionam uma ampla gama de benefícios nas plantas hospedeiras (HUNGRIA, 2011). Esses

microrganismos estimulam o crescimento vegetal por meio de vários processos, tais como: fixação biológica de nitrogênio (HUERGO et al., 2008); aumento da atividade da enzima nitrato redutase (CASSÁN; SALAMONE, 2008); pela síntese de hormônios vegetais, como auxinas, citocininas (TIEN; GASKINS; HUBBELL, 1979), giberelinas (BOTTINI et al., 1989) e etileno (STRZELCZYK; KAMPER, 1994); capacidade de solubilização de fosfato (RODRIGUEZ et al., 2004); além de exercer controle biológico de patógenos (CORREA et al., 2008).

As BPCP mais estudadas atualmente pertencem ao gênero *Azospirillum*, devido a sua aplicabilidade como inoculante no Brasil e na Argentina para gramíneas. Diversos estudos têm comprovado efeitos benéficos do *Azospirillum* em culturas como o trigo, milho e arroz (DOBBELAERE et al., 2002; RODRIGUES et al., 2008; HUNGRIA et al., 2010; HUNGRIA, 2011; MARKS et al., 2013). Além disso, estudos mais recentes indicam que esses microrganismos também são benéficos quando coinoculados com rizóbios em plantas leguminosas, como a soja e o feijoeiro (HUNGRIA; NOGUEIRA; ARAUJO, 2013; CHIBEBA et al., 2015; HUNGRIA; NOGUEIRA; ARAUJO, 2015)

Entre as práticas sustentáveis adotadas na agricultura, a coinoculação de BPCPs compatíveis com rizóbios se mostra promissora (HUNGRIA; NOGUEIRA; ARAUJO, 2013; CHIBEBA et al., 2015; CERZINI et al., 2016), como estratégia para estimular a nodulação inicial e a atividade da FBN, promover o crescimento e aumentar o potencial produtivo em várias leguminosas (LIMA et al., 2011; CHIBEBA et al., 2015; HUNGRIA; NOGUEIRA; ARAUJO, 2015; CERZINI et al., 2016), além de amenizar os efeitos negativos da restrição hídrica moderada em soja (HUNGRIA; NOGUEIRA; ARAUJO, 2015; CERZINI et al., 2016). Isso se deve, entre outros fatores, à capacidade das BPCPs de produzir fitormônios que promovem maior desenvolvimento do sistema radicular, possibilitando maior exploração do solo (LIMA et al., 2011; SAHARAN; NEHRA, 2011) que resulta em maior eficiência na absorção de água e nutrientes. Efeitos benéficos da coinoculação com BPCP também foram observados em condições de estresse salino e limitação nutricional (EGAMBERDIEVA et al., 2010; EGAMBERDIEVA et al., 2013).

A coinoculação de *Rhizobium* e *Pseudomonas spp.* em plantas de galega (*Galega orientalis* Lam.) estimulou o crescimento, a nodulação, aumentou o teor de N na parte aérea e amenizou o estresse salino em relação à inoculação

apenas com *Rhizobium* (EGAMBERDIEVA et al., 2010). Essas contribuições estão relacionadas, entre outros fatores, com o aumento na absorção de água e nutrientes pela ampliação do sistema de raízes promovida pelas BPCPs.

A coinoculação de rizóbios via sementes e *Azospirillum brasilense* via sulco aumentou a produtividade em 16,1% para a soja e 19,6% para o feijoeiro, quando comparados ao tratamento sem inoculação (HUNGRIA; NOGUEIRA; ARAUJO, 2013); com relação ao tratamento que recebeu exclusivamente inoculação com rizóbios, os incrementos foram de 7,1% e 14,7%, respectivamente. Em outro estudo, a coinoculação com *Bradyrhizobium* spp. com *Azospirillum brasilense* nas sementes aumentou o rendimento de grãos em soja em relação aos tratamentos que receberam somente inoculação com *Bradyrhizobium*, ou aqueles suplementados com N mineral (HUNGRIA; NOGUEIRA; ARAUJO, 2015). Chibeba et al. (2015) estudaram o efeito da coinoculação com *Bradyrhizobium* spp. e *Azospirillum brasilense* na nodulação inicial e na FBN em soja, em experimentos em casa de vegetação e a campo e observaram que a coinoculação estimula a nodulação precoce, o que possibilita que os benefícios da FBN também sejam alcançados mais precocemente.

2.4.3 Metabólitos Secundários Microbianos: Oligossacarídeos Lipoquitínicos (LCOs)

O metabolismo primário é denominado o conjunto de reações químicas e processos catabólicos e anabólicos que são essenciais ao crescimento celular e altamente conservados entre as espécies, gêneros e reinos (SIMÕES et al., 2010; O'BRIEN; WRIGHT, 2011). No entanto, algumas plantas e diversos microrganismos são capazes de produzir substâncias denominadas metabólitos secundários, que não necessariamente estão relacionadas à manutenção da vida do organismo produtor (SIMÕES et al., 2010), por não serem essenciais ao crescimento e à reprodução do organismo (MADIGAN et al., 2008). Essas substâncias apresentam, na maioria das vezes, estruturas químicas diferentes daquelas encontradas nos produtos primários do metabolismo e envolvem funções distintas do metabolismo primário (O'BRIEN; WRIGHT, 2011).

Em geral, os metabólitos secundários são compostos por moléculas orgânicas complexas, cuja síntese é realizada em células especializadas, em pequenas quantidades e requer um grande número de reações enzimáticas

específicas (MADIGAN et al., 2008). Apesar de não atuarem diretamente no crescimento e desenvolvimento do organismo, essas substâncias exercem distintas funções, como defesa contra agentes externos, atuam como hormônios, estimulam a formação de simbioses, promovem crescimento microbiano e de plantas, entre outros (DAVIES, 1992). Assim, a síntese desses metabólitos garantem vantagens para sua sobrevivência e para a perpetuação da espécie em seu ecossistema (SIMÕES et al., 2010).

Dentre os metabólitos secundários microbianos (MSM) dos rizóbios, destacam-se os fatores microbianos de nodulação (Nod), identificados como oligossacarídeos lipoquitínicos (LCOs), moléculas essenciais para comunicação e estabelecimento da simbiose entre rizóbios e leguminosas (GOUGH, 2003). Esses compostos são sintetizados pelo microrganismo e liberados no ambiente circunvizinho, onde são reconhecidos por receptores específicos localizados na epiderme da raiz da planta hospedeira e induzem uma variedade de sinalizações genéticas e metabólicas que dão início ao processo de infecção, com encurvamento dos pelos radiculares e formação dos cordões de infecção (KUNERT et al., 2016). O processo de nodulação é extremamente complexo, com a participação de diversas moléculas-sinais (ZAYAS, 2005), que permitem que as primeiras fases de formação dos nódulos ocorram corretamente e torne a simbiose funcional.

Os MSM purificados têm ação em raízes de leguminosas mesmo em concentrações muito baixas, induzindo várias respostas induzidas pelos rizóbios (GOUGH, 2003). Marks et al. (2013) observaram que a adição de MSM na dose de 1 mL L⁻¹ de inoculante em soja cultivada em vasos de Leonard, promoveu aumento de 21% e 12% no número e massa de nódulos, respectivamente, em relação ao tratamento que recebeu apenas inoculação com *Bradyrhizobium*. Em experimentos a campo, a adição de metabólitos secundários na dose de 0,1 mL L⁻¹ de inoculante, aumentou em 23,6% o número de nódulos e em 4,8% o rendimento de grãos de soja em relação ao tratamento que recebeu somente inoculação com *Bradyrhizobium* (MARKS et al., 2013).

Os LCOs, por atuarem como principais moléculas sinalizadoras da simbiose e conferirem especificidade bactéria-hospedeiro pelo envolvimento na infecção e desenvolvimento precoce dos nódulos (CULLIMORE; RANJEVA; BONO, 2001), são moléculas promissoras para aumentar a nodulação. Além disso, tem sido relatado que LCOs podem promover o crescimento das plantas não leguminosas,

resultando em aumento da germinação e resistência a patógenos (MIRANSARI; SMITH, 2009; MARKS et al., 2015).

Uma nova geração de inoculantes enriquecidos com MSM tem se mostrado eficiente em leguminosas, por favorecer a nodulação, desenvolvimento de raízes laterais, aumento da colonização micorrízica, aumento da tolerância a estresses ambientais e do rendimento da cultura (MARKS et al., 2013; SMITH et al., 2015; DEL CERRO et al., 2015; CERZINI et al., 2016). Além disso, os MSM possuem potencial biotecnológico para serem empregados em produtos para pulverização foliar e inoculação de sementes de plantas não-leguminosas (MARKS et al., 2015; SMITH et al., 2015).

Considerando a importância da FBN para a cultura da soja cultivada no Brasil e considerando as ameaças das mudanças climáticas globais ao processo simbiótico e à produtividade da soja, três hipóteses foram testadas nesse estudo: (1) Existem variações genótípicas em soja quando à capacidade de tolerar períodos de seca, em relação à fisiologia da planta e ao processo de FBN, que podem aumentar a estabilidade produtiva da cultura; (2) A capacidade de recuperação da planta após restrição hídrica pode não estar relacionada à capacidade de tolerar a seca; (3) Estratégias que promovam nodulação mais precoce, abundante e funcional pela coinoculação com *Azospirillum* e utilização de MSM, podem maximizar o processo de FBN em soja e conferir vantagem às plantas de soja em condições de seca.

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3 ARTIGO A – RESPOSTAS FISIOLÓGICAS E FIXAÇÃO BIOLÓGICA DE NITROGÊNIO ESTÃO RELACIONADAS ÀS CARACTERÍSTICAS DE TOLERÂNCIA À SECA EM SOJA

Resumo

Estratégias adaptativas das plantas e manutenção da fixação biológica de nitrogênio (FBN) durante a seca, combinada com uma capacidade de recuperação após reidratação são características desejáveis em programas de melhoramento de soja (*Glycine max* L. Merrill) para aumentar a estabilidade de produção de grãos. O objetivo deste estudo foi avaliar seis genótipos de soja com base na sua capacidade de manter parâmetros fisiológicos e a FBN sob restrição hídrica durante pleno florescimento e a capacidade de recuperação após reidratação baseado em componentes de rendimento de grãos. Os genótipos utilizados foram: BRS 317, suscetível à seca; R01-581F e R02-1325, FBN tolerante à seca; e os genótipos recombinantes BRB14-207525, BRB14-207526, BRB14-207527 resultantes do cruzamento entre um genótipo comercial e o genótipo PI 471938, com característica de murchamento lento. BRB-26 manteve a taxa fotossintética e mostrou melhor ajuste das trocas gasosas após 10 dias sob restrição hídrica. A resposta adaptativa do genótipo R02-1325 consistiu em um aumento de 3,5 vezes no sistema radicular. Além disso, R02-1325 e R01-581F não acumularam ureídeos em folhas sob seca, enquanto que BRB-27 minimizou o acúmulo de ureídeos nas folhas, característica reconhecida como FBN tolerante à seca. BRB-27, R02-1325 e BRB-26 mostraram melhor desempenho fisiológico durante a exposição à seca e maior capacidade de rendimento de grãos após a reidratação. As progênies BRB-27 e BRB-26 herdaram traços de tolerância à seca do parental PI 471938, enquanto que R02-1325 é promissor para melhoramento de soja com a finalidade de aumentar a tolerância da soja à seca.

Palavras-chave: Fotossíntese, glutamina sintetase, reidratação, ureídeos.

Physiological responses and biological nitrogen fixation are related to drought tolerance traits in soybean

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Abstract

Plant adaptive strategies and maintenance of biological nitrogen fixation (BNF) during drought stress, combined with a recovery capacity after rewetting are desirable traits in soybean (*Glycine max* L. Merrill) breeding programs to increase the grain yield stability. The aim of this study was to evaluate six soybean genotypes based on their ability to sustain physiological and BNF parameters under drought stress at full flowering, and the recovery capacity after rewetting based on grain yield components. The genotypes were: BRS 317, drought susceptible; R01-581F and R02-1325, nitrogen fixation drought tolerant; and the recombinant genotypes BRB14-207525, BRB14-207526, BRB14-207527 resulting from breeding between commercial genotypes and PI 471938, slow wilting. BRB-26 kept the photosynthetic rate and showed better adjustment of gas exchanges after 10 days under water stress. The adaptive response of R02-1325 consisted of a 3.5-fold increase in root biomass. Moreover, R02-1325 and R01-581F did not accumulate ureides in leaves under water stress, whereas BRB-27 minimized the content of ureides in leaves, a trait recognized as nitrogen fixation drought tolerance. BRB-27, R02-1325 and BRB-26 showed better physiological performance during drought exposure and higher yield capacity after rewetting. The progenies BRB-27 and BRB-26 inherited drought tolerance traits from PI 471938, whereas R02-1325 is promising for breeding aiming at increasing soybean tolerance to drought.

Keywords: glutamine synthetase, photosynthesis, re-wetting, ureides.

1. Introduction

Extreme climate events have often been affected the agricultural production (GUSSO et al., 2014; SHANKER et al., 2014). Several models have

predicted reduction of rainfall and increase of temperatures by the second half of the 21st century (IPCC, 2014, COOK; AULT; SMERDON, 2015), causing severe agricultural losses in crops with high economic importance, such as soybean (*Glycine max* L. Merrill). Facing the global climate changes and the need to keep the food supply (SHANKER et al., 2014), researchers have strived to breed new crop genotypes more stable in keeping the grain yield under drought (DEVI; SINCLAIR, 2013; CEREZINI et al., 2014; DEVI et al., 2014).

Important traits related to drought tolerance were identified in soybean genotypes, as limited transpiration under high vapor pressure deficit in PI 416937 (SINCLAIR; ZWIENIECKIB; HOLBROOK, 2008; DEVI et al., 2014) and slow-wilting in PI 471938 (SADOK et al., 2012; DEVI; SINCLAIR, 2013), collected in Japan and Nepal, respectively. The genotype Jackson presents a rare trait: nitrogen fixation drought tolerance (NFDT) (SALL; SINCLAIR, 1991). The progenies from Jackson, R01-581F (PI 647961) and R01-416F (PI 647960) also showed good performance under drought in the parameters of NFDT and grain yield in experiments performed under in greenhouse and in the field conditions (SINCLAIR et al., 2007; CHEN et al., 2007).

The capacity of limiting water use may save water for critical developmental stages, and thus ensure crop yield (SINCLAIR et al., 2010). Moreover, NFDT in soybean is a key trait to keep the symbiotic process and help to cope with dry conditions by providing the N required by plant (SINCLAIR; VADEZ, 2012). In soybean, the sensibility of BNF under drought is associated with a failure in the transport and metabolism of N compounds, mainly ureides, resulting in accumulation in nodules, petioles and leaves (SALL; SINCLAIR, 1991; SERRAJ; SINCLAIR; PURCELL, 1999; CEREZINI et al., 2014), and causing a feedback inhibition of BNF. However, NFDT soybean genotypes have less accumulation of ureides under drought as compared with sensitive ones (SERRAJ; SINCLAIR; PURCELL, 1999; DEVI; SINCLAIR, 2013; CEREZINI et al., 2014).

Besides the ability of sustaining the BNF and physiological processes as transpiration and photosynthesis during soil drying, the recovery capacity of these processes after drought is important to ensure plant performance (JAMES; LAWN; COOPER, 2008; ROSAS-ANDERSON et al., 2014; CEREZINI; RIAR; SINCLAIR, 2016). Thus, the soybean capacity to recover its physiological activity after re-watering is a fundamental trait to allow stability of grain yield after

moderate drought and is an important trait for genotypic selection aiming at drought tolerance (FELLOWS et al., 1987; CHAVES; MAROCO; PEREIRA, 2003).

The objective of this study was to evaluate the capacity of six soybean genotypes to sustain plant metabolism during soil drying, as well as their recovery capacity to keep grain yield after re-watering. Changes in N metabolism were also assessed to characterize traits related to NFDT, hoping to contribute in breeding programs aiming at improving soybean tolerance to water limitation.

2. Material and Methods

Six soybean genotypes were assessed in a trial performed under controlled conditions at Embrapa Soybean, in the municipality of Londrina, Paraná State, Brazil. The commercial genotype 'BRS 317' is considered drought susceptible (CEREZINI et al., 2014); the genotypes 'R01-581F' (CHEN et al., 2007) and 'R02-1325' (DEVI et al., 2014) were previously identified and selected for NFDT; and three recombinant genotypes ('BRB14-207525', 'BRB14-207526', 'BRB14-207527') are descendant from breeding of commercial genotypes with 'PI 471938', known to exhibit the slow-wilting trait (DEVI; SINCLAIR, 2013) (Table 1). The experimental design was randomized blocks, in 6 x 2 factorial arrangement, i.e., six soybean genotypes and two water conditions: well-watered (WW) and exposed to water restriction (WR) between 35 and 45 days after emergence (DAE), with 10 replicates.

Table 1. Maturity group and pedigree of soybean genotypes used in experiment.

Soybean genotype	Maturity group	Pedigree	Characteristic
BRS 317	6.6	-	Commercial cultivar
R01-581F	5	Jackson x KS 4895	NFDT
R02-1325	5	93705-35-1 x PI 227557	NFDT
BRB14-207525	6.3	PI 471938 x Fonte BtRR2 – P	Slow-wilting
BRB14-207526	6.3	PI 471938 x Fonte BtRR2 – P	Slow-wilting
BRB14-207527	6.3	PI 471938 x Fonte BtRR2 – P	Slow-wilting

NFDT – Nitrogen fixation drought tolerant.

Samples taken at 0-20 cm from an agricultural soil classified as Typic

Acrudox (USDA soil taxonomy) were used as substrate in this experiment. The chemical and texture analyses revealed: pH (CaCl₂) = 5.81; organic matter = 31.4 g kg⁻¹; P (Mehlich I) = 2.85 mg dm⁻³; K = 0.10 cmol_c dm⁻³, Ca = 3.47 cmol_c dm⁻³, Mg = 1.10 cmol_c dm⁻³, H + Al = 4.12 cmol_c dm⁻³, CEC = 8.79 cmol_c dm⁻³, SB = 4.67, V = 53%, Al = 0 cmol_c dm⁻³; particle size fractions: sand = 732 g kg⁻¹, silt = 30 g kg⁻¹, clay = 238 g kg⁻¹. Four-kg aliquots of the substrate were placed into plastic pots and, before sowing, each pot received 54 mg of P, 320 mg of K (both as K₂HPO₄), 230 mg of Mg, 310 mg of S (both as MgSO₄·7H₂O), and 150 mL of micronutrient solution containing 2.85 mg of CoSO₄, 10.8 mg of Na₂MO₄ and 1.0 g of H₃BO₃ in 15 L of water. Nitrogen was provided via BNF, by inoculation with a mixture of *Bradyrhizobium japonicum* (strain SEMIA 5079) and *B. diazoefficiens* (strain SEMIA 5080), as recommended in commercial inoculants in Brazil, just before sowing, using a broth containing 1×10⁹ viable cells mL⁻¹ and supplying each seed with 0.5 mL. Each pot received six seeds that were thinned to two plants per pot one week later.

For soil moisture maintenance, the water-holding capacity was determined by the tension table and Richards's extractor device (EMBRAPA, 1997), which allowed the construction of a water-retention curve and determination of soil water potential (ψ_w). During the first 35 DAE, plants received water daily to maintain the ψ_w at -13 kPa (300 mL of water per L of soil), which represent a fraction of available water (FAW) of 0.9. After 35 DAE, during the flowering stage, plants were subjected to water restriction for 10 days at -200 kPa (90 mL of water per L of soil), with a FAW of 0.27. The control plants continued to receive normal water supply at -13 kPa of ψ_w . Soil moisture was monitored daily by weighing the pots on an electronic scale, with replacement of water during the morning (between 8-10 h a.m.). During the experiment, daily temperatures and relative air humidity were measured with a thermohygrometer. The average night/day temperatures ranged between 21.7 °C and 33.5 °C, respectively, and the average day/night relative humidity ranged from 42.0% to 81.4%, respectively.

During the stress period (35-45 DAE), SPAD units were obtained between 8-10 h a.m. in the third recently expanded trifoliolate with a portable meter, model SPAD-502 (Konica Minolta Sensing Inc., Osaka, Japan), and converted into leaf-chlorophyll concentration ($\mu\text{g cm}^{-2}$) (KASCHUK et al., 2010). Physiological parameters were measured in both stressed and non-stressed plants at the 5th and 10th days under water restriction, with a portable gas-exchange meter (LCpro-SD,

ADC BioScientific Ltd., UK). Determinations included net photosynthetic (A) and transpiration rate (E), stomatal conductance (g_s), intercellular CO_2 concentration (c_i) and temperature of leaves (T_{leaf}). Gas exchanges were also performed in the third recently expanded trifoliolates in the morning (9-11 h a.m.).

After the water restriction period, six out of 10 replicates were harvested for analysis. Roots and nodules samples were immediately frozen in liquid N_2 and stored at $-80\text{ }^\circ\text{C}$ for assessment of glutamine synthetase (GS) activity, after extraction using a Sephadex G-25 column (HUNGRIA, 1994), and determination according to Ferguson and Sims (1971). Shoots and the remaining roots with nodules were dried at $60\text{ }^\circ\text{C}$ for 48 h for determining the shoot and root dry weight (SDW and RDW), number of nodules (NN) and nodule dry weight (NDW); the total shoot N content (TSNC) were determined in sulfuric extracts by the green salicylate colorimetric method (SEARLE, 1984). The ureides content was determined in dry nodules, petioles and leaves after extraction according to Hungria (1994) and colorimetric determination (VOGELS; VAN DER DRIFT, 1970). The remaining four replicates were kept under normal water supply until the physiological maturity to obtain the grain yield. At that time, plants were assessed for number of pods, number and weight of grains (12% humidity), protein and oil content in grains by Near-infrared (NIR) using the Antaris II FT-NIR spectrophotometer (Thermo Electron Co., USA).

The dataset was submitted to tests of normality and homogeneity of variances followed by ANOVA with application of F test at $p \leq 0.05$. Once the effects of treatments or interactions between factors were detected, means were compared by Tukey's test at $p \leq 0.05$.

3. Results

Water restriction affected negatively the physiological parameters in both assessment dates (Table 2); at the fifth day under WR, there was a general reduction of net photosynthetic and transpiration rates, by 11% and 15%, respectively, in the average of genotypes. The stomatal conductance and CO_2 concentration also reduced by 41% and 17%, respectively. Finally, the temperature of leaves increased by $1\text{ }^\circ\text{C}$ in plants under WR.

Table 2. Net photosynthetic and transpiration rates, stomatal conductance, intercellular CO₂ concentration and temperature of leaves in six soybean genotypes under water restriction (WR) or well-watered (WW), between 35 and 45 days after emergence.

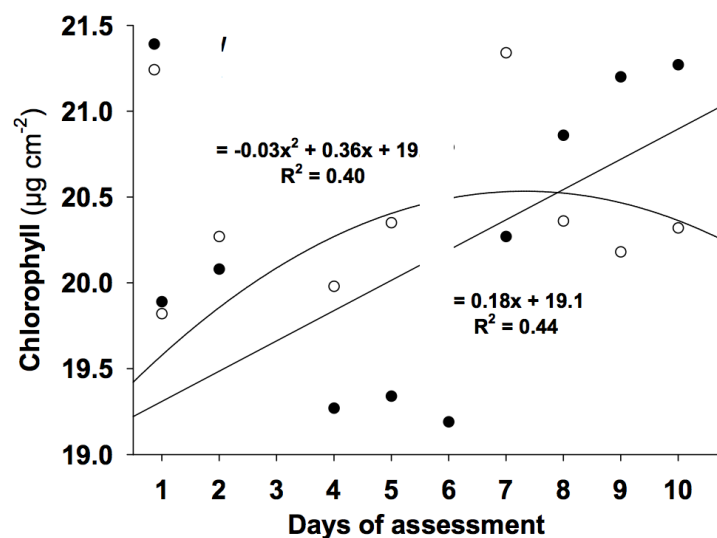
Genotypes	5 days under water restriction			10 days under water restriction		
	WW	WR	Average	WW	WR	Average
Net photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)						
BRS 317	20.3	18.2	19.3	17.91 Aa	12.1 Bbc	15.00
R01-581F	22.7	19.0	20.9	21.85 Aa	12.9 Bbc	17.37
R02-1325	20.7	17.5	19.1	21.35 Aa	11.1 Bc	16.21
BRB-25	20.8	21.2	21.0	20.42 Aa	15.4 Bab	17.90
BRB-26	23.3	20.1	21.7	19.69 Aa	17.0 Aa	18.36
BRB-27	22.6	19.8	21.2	20.93 Aa	14.9 Babc	17.89
Average	21.7 A	19.3 B		20.36	13.9	
Transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)						
Genotypes	WW	WR	Average	WW	WR	Average
BRS 317	3.69	3.28	3.49 b	3.33 Ab	1.91 Bbc	2.62
R01-581F	4.11	3.29	3.70 ab	3.94 Aab	2.26 Babc	3.10
R02-1325	3.68	3.19	3.44 b	3.61 Aab	1.74 Bc	2.68
BRB-25	4.18	3.83	4.01 a	4.06 Aa	2.58 Ba	3.32
BRB-26	4.01	3.66	3.83 ab	3.65 Aab	2.78 Ba	3.22
BRB-27	4.67	3.54	4.11 a	4.10 Aa	2.51 Bab	3.31
Average	4.06 A	3.47 B		3.78	2.30	
Stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)						
Genotypes	WW	WR	Average	WW	WR	Average
BRS 317	0.439	0.244	0.342 c	0.292 Ab	0.083 Bbc	0.188
R01-581F	0.458	0.270	0.364 bc	0.362 Aa	0.097 Bbc	0.230
R02-1325	0.427	0.225	0.326 c	0.386 Aa	0.078 Bc	0.232
BRB-25	0.468	0.341	0.405 abc	0.403 Aa	0.135 Bab	0.269
BRB-26	0.538	0.343	0.441 ab	0.367 Aa	0.181 Ba	0.274
BRB-27	0.602	0.313	0.458 a	0.401 Aa	0.133 Bab	0.267
Average	0.489 A	0.289 B		0.369	0.118	
Intercellular CO₂ concentration ($\mu\text{mol CO}_2 \text{ mol}^{-1}$)						
Genotypes	WW	WR	Average	WW	WR	Average
BRS 317	302	250	276	280	162	221 b
R01-581F	299	230	264	268	201	234 ab
R02-1325	288	227	258	273	157	215 b
BRB-25	299	259	279	282	207	245 ab
BRB-26	297	272	285	286	214	250 a
BRB-27	302	247	275	280	191	236 ab
Average	298 A	248 B		278 A	189 B	
Temperature of leaves ($^{\circ}\text{C}$)						
Genotypes	WW	WR	Average	WW	WR	Average
BRS 317	29.1	30.3	29.7	29.1	31.2	30.1 ab
R01-581F	29.8	30.8	30.3	29.7	32.1	30.9 a
R02-1325	29.1	30.4	29.8	29.1	31.4	30.2 ab
BRB-25	29.5	30.4	29.9	29.6	31.5	30.6 a
BRB-26	29.1	29.8	29.5	29.1	30.0	29.5 b
BRB-27	29.7	30.5	30.1	29.5	31.4	30.5 ab
Average	29.4 B	30.4 A		29.3 B	31.3 A	

Means followed by the same letter, uppercase in lines and lowercase in columns, do not differ statistically one another (Tukey, $p < 0.05$) ($n = 10$).

At the tenth day, the water condition affected differentially each genotype concerning net photosynthetic and transpiration rates, and stomatal conductance. BRB-26 did not show reduction of photosynthesis under WR, and showed the highest photosynthetic rate in relation to BRS 317, R01-581F and R02-1325 genotypes, which showed reduction of 40% under WR. The transpiration rate was significantly reduced in all genotypes under WR, but BRB-25 and BRB-26 showed higher transpiration rates than BRS 317 and R02-1325. Moreover, BRB-25 and BRB-26 reduced transpiration by 36% and 24%, respectively, whereas BRS 317 and R02-1325 had reductions of 43% and 52%, respectively. The stomatal conductance was also reduced in all genotypes, but once again BRB-26 showed better performance under WR, with reductions of 51% of stomatal conductance whereas the other genotypes show an average reduction of 72% compared with plants well-watered.

Intercellular CO₂ and temperature of leaves showed only effect of water supply at 10 days under WR, with decrease of 32% and increase of 2 °C, respectively. The chlorophyll content decreased in the last days in plants under WR, whereas the WW plants showed a positive linear increase in chlorophyll content along the assessment (Fig. 1).

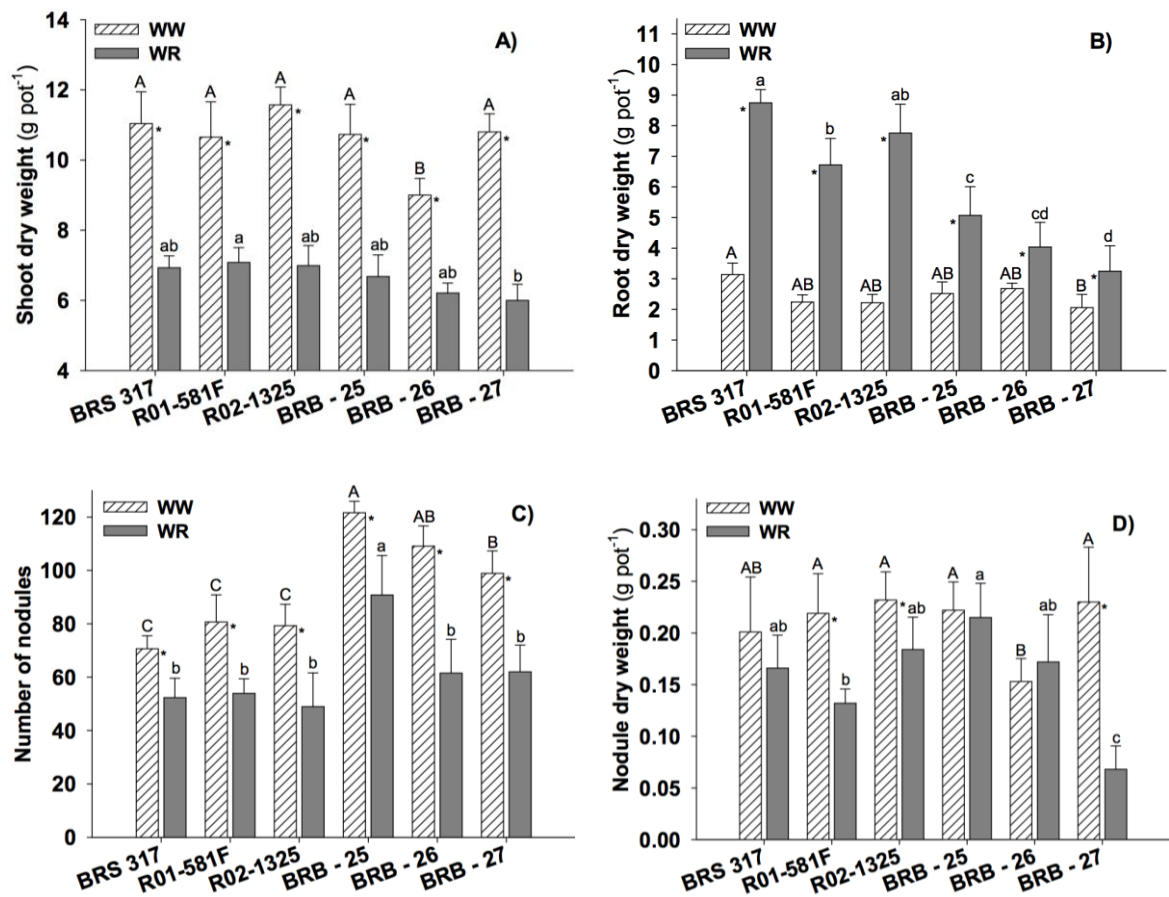
Figure 1 - Average chlorophyll content in six soybean genotypes (BRS 317, R01-581F, R02-1325, B14-207525, BRB14-207526, BRB14-207527) under either well-watered (WW) or water restriction (WR) conditions, between 35 and 45 days after emergence (n = 10).



Water restriction reduced the SDW of 37% in the average of genotypes in relation to well-watered plants (Fig. 2A), in which R01-581F showed higher SDW in relation to BRB-27. On the other hand, RDW increased with WR in all genotypes (Fig. 2B). R02-1325 showed the highest relative increase, 3.5 more than the WW control. BRS 317 showed the highest absolute value, whereas BRB-27 the lowest.

Higher NN was observed in BRB-25 in both water conditions (Fig. 2C). Although WR reduced NN in all genotypes, BRB-25 showed almost 40% more nodules than the other genotypes. BRS 317, BRB-25 and BRB-26 kept the NDW under WR, whereas the other genotypes had reductions compared with well-watered plants (Fig. 2D). BRB-25 showed more NDW under WR than R01-581F and BRB-27.

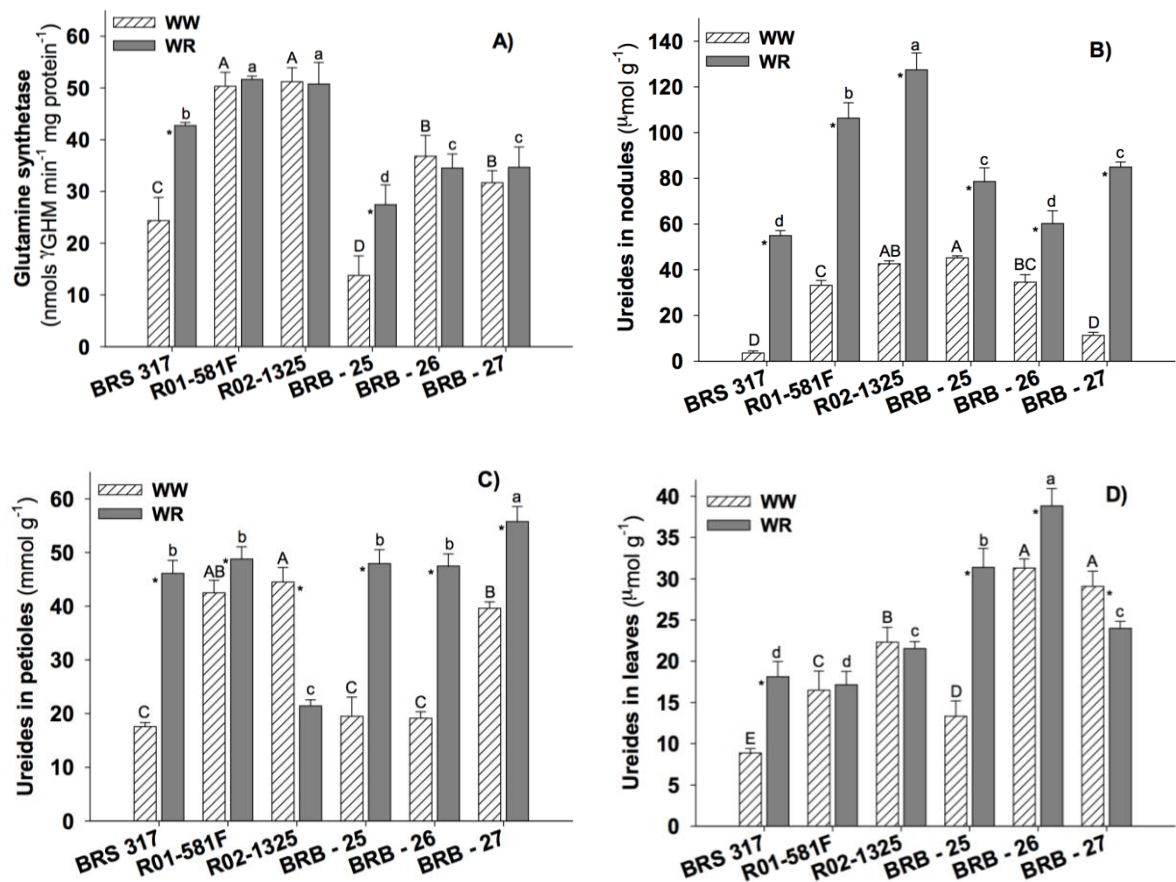
Figure 2. Shoot dry weight (A), root dry weight (B), number of nodules (C) and nodule dry weight (D) in six soybean genotypes (BRS 317, R01-581F, R02-1325, B14-207525, BRB14-207526, BRB14-207527) under well-watered (WW) or water restriction (WR), between 35 and 45 days after emergence.



Means followed by the same letter, uppercase for WW and lowercase for WR, do not differ one another (Tukey, $p < 0.05$); *Significant difference between water condition in each genotype. Vertical bars represent the standard deviation (SD) ($n = 6$).

GS enzyme activity was higher in R01-581F and R02-1325 in both water conditions (Fig. 3A). These genotypes, along with BRB-26 and BRB-27, kept the enzyme activity under WR, whereas BRS 317 and BRB-25 increased the activity. Ureides in nodules had an expressive increase under WR in all genotypes (Fig. 3B). The highest concentration was observed in R02-1325 followed by R01-581F, resulting in 60% more ureides in nodules than the average of the remaining genotypes. However, BRS 317 showed the highest relative increase of ureides in nodules, 15 times more than the counterparts under WW condition did.

Figure 3 - Glutamine synthetase activity in nodules (A), concentrations of ureides in nodules (B), petioles (C) and leaves (D) in six soybean genotypes (BRS 317, R01-581F, R02-1325, B14-207525, BRB14-207526, BRB14-207527) under well-watered (WW) or water restriction (WR), between 35 and 45 days after emergence.



Means followed by the same letter, uppercase for WW and lowercase for WR, do not differ one another (Tukey, $p < 0.05$); *Significant difference between water condition in each genotype. Vertical bars represent the standard deviation (SD) ($n = 6$).

Ureides in petioles also increased for most genotypes under WR (Fig. 3C). Conversely, R02-1325 showed reduction by 52% under WR, but showed the highest concentration under WW condition, not differing from R01-581F. The genotype BRB-27 had the highest concentration under WR. Considering the relative increase compared with the WW plants, R01-581F increased only 15% under WR, whereas the average increase in most of genotypes (BRS 317, BRB-25, BRB-26 and BRB-27) was more than 120%.

Concentration of ureides in leaves increased by 104%, 135% and 24% under WR for BRS 317, BRB-25 and BRB-26, respectively, compared with WW plants (Fig. 3D). R01-581F and R02-1325 did not show significant increase, whereas BRB-27 reduced it by 17%. The highest concentration of ureides in leaves under WW condition were observed in BRB-26 and BRB-27; under WR, BRB-26 showed the highest concentration.

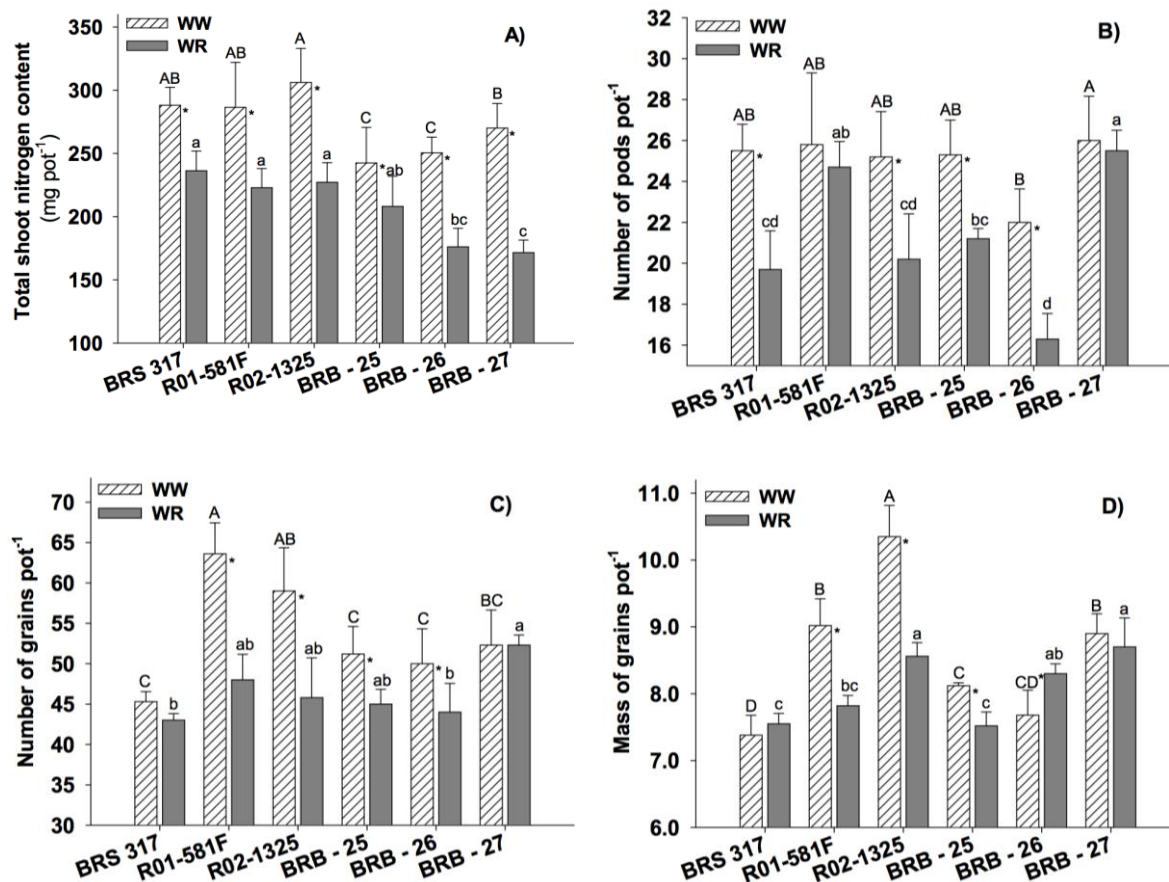
All plants showed reduction of TSNC under WR (Fig. 4A) irrespectively of the genotype. At WW condition, R02-1325 showed greater N content in shoot, not differing from BRS 317 and R01-581F. Under WR, BRS 317, R01-581F and R02-1325 showed higher contents in relation to BRB-26 and BRB-27.

The water restriction reduced the number of pods by 20% in BRS 317, R02-1325, BRB-25 and BRB-26 (Fig. 4B), but R01-581F and BRB-27 showed no reductions. BRB-27 produced more pods in both water conditions, but not differing from R01-581F under WR, and differing only from BRB-26 under WW condition. Only BRS 317 and BRB-27 genotypes kept the number of grains after exposure to drought (Fig. 4C) compared with the WW counterparts. R01-581F, R02-1325, BRB-25 and BRB-26 reduced the number of grains by 25%, 22%, 12% and 12%, respectively, after exposure to WR. R01-581F had the highest number of grains when WW, not differing from R02-1325. Under WR, BRB-27 produced more grains compared with BRS 317 and BRB-26.

The water condition did not affect the mass of grains of BRS 317 and BRB-27; R01-581F, R02-1325 and BRB-25 show reductions of 13%, 17% and 7% after exposure to WR, respectively. However, BRB-26 increased the mass of grains by 8% after exposure to WR between 35-45 DAE (Fig. 4D). R02-1325 and BRB-27 genotypes had the highest mass of grains when exposed to WR, not differing from BRB-26. Protein and oil content in grains varied between genotypes, but there was

no effect of water condition. R02-1325 and BRB-25 showed the highest protein contents in grains, whereas BRB-27 had higher oil contents compared with R01-581F, R02-1325 and BRB-25 (data not shown).

Figure 4. Total shoot nitrogen content (A) after 10 days of drought stress, number of pods (B), number of grains (C) and mass of grains (D) in six soybean genotypes (BRS 317, R01-581F, R02-1325, B14-207525, BRB14-207526, BRB14-207527) under well-watered and drought stress conditions, between 35 and 45 days after emergence.



Means followed by the same letter, uppercase for WW and lowercase for WR, do not differ one another (Tukey, $p < 0.05$); *Significant difference between water condition in each genotype. Vertical bars represent the standard deviation (SD) ($n = 6$).

4. Discussion

Drought stress is one of the greatest environmental threats affecting soybean yield (LAUER et al., 2012; DEVI et al., 2014; HURA et al., 2015; CERZINI et al., 2016). The extent of losses depends on the intensity and duration of drought,

plant developmental stage, plant capacity to tolerate drought, and recovery capacity after rehydration (SINCLAIR et al., 2007; MASTRODOMENICO; PURCELL; KING, 2013; CEREZINI et al., 2014, DEVI et al., 2014; SHANKER et al., 2014; HURA et al., 2015; CEREZINI, RIAR, SINCLAIR, 2016). Thus, drought tolerance traits are useful to find and understand the most favorable features that ensure plant yield, under a broader range of environmental limitation, which can be used in selection programs aiming at soybean tolerance to drought.

In our study, the duration of drought stress intensified the effects on the gas exchanges. With five days under water restriction, there was reduction in stomatal conductance (Table 2) and other physiological variables, featuring a mild stress. Five days later, all parameters evaluated reduced even more, reaching a higher stress level. As the stress level can be measured by the stomatal conductance, non-stressed plants usually have $g_s \geq 0.2 \text{ molH}_2\text{O m}^{-2} \text{ s}^{-1}$, moderate drought stress results g_s between $0.1\text{-}0.2 \text{ molH}_2\text{O m}^{-2} \text{ s}^{-1}$, whereas $g_s \leq 0.1 \text{ molH}_2\text{O m}^{-2} \text{ s}^{-1}$ evidences a severe drought stress (FLEXAS et al., 2004). Based on that scale, only the genotypes BRS 317, R01-581F and R02-1325 reached a severe stress level after 10 days under water restriction, whereas BRB-25, BRB-26 and BRB-27, although under the same water condition, showed a moderate stress level.

The maintenance of gas exchanges under drought stress is a characteristic probably inherited from the parental 'PI 471938'. Although 'PI 471938' show slow-wilting phenotype under drought, it does not show early decrease in stomatal conductance under drought in order to save water in soil (SADOK et al., 2012; DEVI; SINCLAIR, 2013). Thus, this hypothesis cannot explain the slow-wilting phenotype, and the basis for drought tolerance in this genotype remains unknown (SADOK et al., 2012).

BRB-26 was the most outstanding genotype able to keep the physiological parameters after 10 days of drought stress, with maintenance of net photosynthetic rate and good performance of transpiration, stomatal conductance and leaf temperature. However, there was no advantage on shoot biomass, which decreased for all genotypes under drought, featuring an adaptive strategy of plants to reduce the loss of water. Decrease in stomatal conductance and consequently decline in transpiration, CO_2 assimilation, chlorophyll content, and increase in leaf temperature are the most clear plant responses under soil drying (CHRISTOPHE et al., 2011; MUTAVA et al., 2015). The combination of these factors lead to decrease

in shoot dry weight and stimulate the elongation of root system into deeper soil layers to find water (LOPES et al., 2011; CERZINI et al., 2014). R02-1325 showed higher capacity to increase of the root system under drought, a useful trait in the selection for drought tolerant genotypes (LOPES et al., 2011).

Exposure to drought quickly affected the nodulation and N metabolism, including ureides and N accumulation. The formation of nodules is an expensive process in terms of photosynthates and competes for nutrients with other organs such as roots, leaves and seeds (VOISIN; MUNIER-JOLAIN, SALON, 2010; PRUDENT et al., 2016). The regulation of nodulation would be among several adaptive plant responses to drought (CHRISTOPHE et al., 2011), which would help to ensure the plant survival (VOISIN; MUNIER-JOLAIN, SALON, 2010) by saving photosynthetic resources. Under this viewpoint, BRB-25 stood out based on its capacity to keep the nodulation under drought, in addition to less reduction in shoot N accumulation as compared with well-watered plants.

Many studies have confirmed the sensibility of BNF to drought (SERRAJ; SINCLAIR; PURCELL, 1999; KING; PURCELL, 2005; SINCLAIR et al., 2007; ARRESE-IGOR et al., 2011; CERZINI et al., 2014; CERZINI et al., 2016) associated to impairment of transport, assimilation and accumulation of N compounds in the plant. The enzyme GS is the most important via (GS-GOGAT) for N assimilation under normal growth conditions (MIFLIN; HABASH, 2002; VANCE, 2008). However, the GS activity decreases with drought (FIGUEIREDO; BEZERRA-NETO; BURITY, 2001; FIGUEIREDO et al., 2007; HUNGRIA; KASCKUK, 2013), and negatively affects the N metabolism.

Despite the GS activity remained stable in R01-581F, R02-1325, BRB-26 and BRB-27 exposed to drought, the concentration of ureides in nodules and petioles increased, that are potential factors for a possible feedback inhibition of the BNF in long-term (DEVI; SINCLAIR, 2013). However, the genotypes R01-581F and R02-1325, considered NFDT (CERZINI et al., 2014; DEVI et al., 2014), kept the concentration of ureides stable in leaves as their parental 'Jackson' (SALL; SINCLAIR, 1991; SERRAJ; SINCLAIR; PURCELL, 1999; CERZINI et al., 2014). Similarly, BRB-27 showed ability to minimize the accumulation of ureides in leaves under drought stress, which could be one more trait to deal with drought tolerance.

Maintenance of BNF during drought stress combined with recovery capacity after drought are desirable traits in soybean that can bring advantages for

grain yield. The BNF process in soybean is restored five days after moderate drought in the vegetative stage, but may take longer if the drought occurs during the reproductive stage (MASTRODOMENICO et al., 2013). In studies of Cerezini, Riar and Sinclair (2016), the transpiration rate and the BNF of soybean in vegetative stage showed fast recovery after severe water restriction followed by re-watering. Our results revealed that the soybean genotypes differ in their ability to recover and keep the yield after exposure to drought during the same phenological stage. Evidences of genetic variation in soybean and peanut (*Arachis glabrata* L.) regarding the recovery capacity of transpiration, stomatal conductance and BNF after soil drying and rehydration has also been described (ROSAS-ANDERSON et al., 2014; CEREZINI, RIAR, SINCLAIR, 2016). In soybean, genotypes with different capacity to sustain the BNF under moderate drought stress during the flowering have also been identified (CHEN et al., 2007; SINCLAIR et al., 2007; CEREZINI et al., 2014), but the effects on grain yield have not been evaluated.

The genotype BRB-27 outstood for keeping all yield components after exposition to drought, resulting in higher grain mass. However, the genotype R02-1325, although have reduced grain mass after drought, still kept yield potential similar to BRB-27 and BRB-26. Genotypes like R02-1325 would be preferable in the field because they have higher potential for grain yield under wet conditions but still keep the yield potential under drought. Comparing grain yield with the capacity to sustain the plant metabolism and BNF under drought stress, the genotypes reveal different mechanisms of adaptation to drought that could help to sustain the grain yield, like the ability to keep the photosynthesis, elongation of root system, maintenance of GS activity and lower concentrations of ureides in leaves. Further research comparing these traits between different soybean genotypes, in addition to the recovery capacity after drought, are targets for breeding soybean in which BRB-26, BRB-27 and R02-1325 could be used as genetic sources of tolerance to drought.

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4 ARTIGO B - CAPACIDADE DE RECUPERAÇÃO DA TRANSPIRAÇÃO E DA FIXAÇÃO BIOLÓGICA DE NITROGÊNIO EM SOJA APÓS SECA

Resumo

A capacidade de recuperação da atividade fisiológica após a reidratação é uma característica fundamental para avaliar o potencial produtivo da cultura da soja após períodos de seca. O objetivo deste estudo foi avaliar a velocidade de recuperação de plantas de soja quanto à taxa transpiratória e atividade da fixação biológica de nitrogênio pelo método da redução de acetileno, submetidas ao déficit hídrico seguido do restabelecimento do fornecimento de água. A recuperação foi analisada em dez genótipos de soja, sendo dois genótipos parentais (PI 471938 e Hutcheson) com diferentes respostas à deficiência hídrica, e oito progênies derivadas do seu cruzamento. Quatro experimentos foram realizados em casa de vegetação com diferentes níveis de tratamentos de seca. A recuperação após o período de seca foi geralmente rápida, mesmo nos tratamentos submetidos à restrição hídrica severa. A taxa transpiratória das plantas foi recuperada, em média, em 2 a 3 dias, enquanto que a fixação do nitrogênio apresentou recuperação média em 2 a 5 dias. Algumas variações genotípicas quanto à capacidade de recuperação foram identificadas, no entanto, essas diferenças foram sutis e provavelmente não representariam um grande impacto no desempenho da cultura no campo.

Palavras-chave: Fixação de nitrogênio, seca, soja, transpiração.

Transpiration and Nitrogen Fixation Recovery Capacity in Soybean Following Drought Stress

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ABSTRACT

The recovery capacity of physiological activity after rewatering is a fundamental trait in assessing crop productivity after periods of drought. The objective of this study was to document the rapidity of recovery of both transpiration rate (TR) and nitrogen fixation (NF) activity by soybean (*Glycine max* (Merr.) L.) plants after being subjected to defined soil water deficits. Recovery was studied for ten genotypes: two parent genotypes (PI 471938, which expressed nitrogen fixation tolerant to water deficit, and Hutcheson, which expresses nitrogen fixation sensitivity to water deficit), and eight progeny genotypes derived from the mating of these parents. This divergence in response to drought of parents and progeny lines potentially offered divergence in rate of recovery from drought stress. Four experiments were conducted in a greenhouse with three different levels of severity in drought treatments. The recovery even under severe drought was generally rapid: TR recovered in 2–3 d and NF recovered in 2–5 d. Some genotypic variation in recovery rate was identified, but these differences were small and were expected not to have a large impact on field performance.

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Drought; drought recovery; nitrogen fixation; soybean; transpiration

Introduction

Negative effects of drought stress on soybean [*Glycine max* (L.) Merr.] have been widely documented (Grinnan, Carter, and Johnson 2013; Sinclair and Vadez 2012; Streeter 2003). Specifically, drought severely affects soybean growth and development and may cause yield loss up to 40% in the worst years (Manavalan et al. 2009; Thao and Tran 2012). One very important aspect of the yield loss is high vulnerability of biological nitrogen fixation (NF) to soil drying (Sprent 1972). Efforts have been made in understanding and selection of soybean genotypes with the ability to sustain physiological process of plants in general, and also the NF process, as soils dry (Sinclair et al. 2007; Cerezini et al. 2014; Devi and Sinclair 2013; King and Purcell 2006; King et al. 2014; Serraj, Sinclair, and Purcell 1999).

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Soybean genotypes PI 416937 and PI 471938 were identified as candidates expressing drought tolerance as visualized by their “slow-wilting” phenotype when subjected to drought. Slow-wilting expressed by PI 416937 was found to be associated with low hydraulic conductance in leaves that result in limited-transpiration rate under high atmospheric vapor pressure deficit. (Sadok and Sinclair 2009; Sinclair, Zwieniecki, and Holbrook 2008). However, PI 471938 does not have the same physiological adjustment as PI 416937. Devi and Sinclair (2013) reported the important discovery that PI 471938 plants were able to sustain NF activity at low soil water content. Additional genotypes have been identified as expressing NF drought-tolerance (Devi and Sinclair 2013; Devi et al. 2014) and also, high yield.

Because soybean is often exposed to at least brief periods of drought, recovery of NF from drought stress is another important response characteristic in understanding of the response of soybean to short periods of drought, and also for genotypic selection (Fellows et al. 1987). However, there is virtually no knowledge about the ability of soybean, or any other grain legume, to recover both transpiration rate (TR) and NF rates after drought. King and Purcell (2005) subjected two genotypes of soybean to rapid soil dehydration (4 d) and found substantial NF recovery with watering during the succeeding 2 d. Mastrodomenico, Purcell, and King (2013) imposed a constant water-deficit in the soil on a single genotype for 5 d and found NF recovery 5 d after watering. Neither of these studies reported TR recovery and the extent of data collected did not allow clear temporal resolution of NF recovery.

Thus, the objective of this study was to document the rapidity in soybean for both TR and NF recovery from soil drying on a daily basis for at least 5 d following rewatering. To expand the genetic base of these observations, 10 soybean genotypes were included in the study. The initial experimental treatment was to subject plants to a substantial water-deficit stress in which plants were severely wilted and transpiration rate was equal to or less than 10% of well-watered plants. In anticipation that the severe water-deficit would result in major damage in the plant resulting in little recovery, two additional experiments were done to include observations on recovery from more modest water-deficit stresses.

Materials and methods

Experiment description

Four experiments were conducted in a greenhouse located at Raleigh, NC (46°35'N, 39°78'W) to evaluate the recovery of TR and NF rate of a total of ten soybean genotypes after drought treatments. The genotypes analyzed included two parent genotypes—PI 471938, identified in the field by the

expression of a “slow-wilting” phenotype and Hutcheson, a fast-wilting cultivar, and eight heterogeneous inbred family (HIF) progeny genotypes descended from the PI 471938 x Hutcheson cross. The progeny genotypes are identified as N05-UGA-848, N05-UGA-852, N05-UGA-854, N05-UGA-862, N05-UGA-863, N05-UGA-875, N05-UGA-886, and N05-UGA-888.

Plants were grown in polyvinyl chloride pots (10-cm diameter and 30-cm tall) filled with loamy soil (69% sand, 18% silt, and 13% clay). The soil had been inoculated with *Bradyrhizobium* resulting in good nodulation of all plants. The plants were kept under well-watered condition until V3-V4 phenological stage, when the dry-down test began. Temperature of the greenhouse was regulated to achieve 28°C for both day and night temperatures.

Because the system for measuring NF activity could only accommodate 40 plants in each experiment, two experiments of five genotypes each were initially tested. This arrangement allowed eight plants of each genotype to be studied in each batch. For Exp. 1 and 2, the pots for each genotype were divided at the initiation of the soil drying treatment between three well-watered pots and five drying pots. Exp. 1 occurred between 1 and 17 July 2015, and Exp. 2 occurred between 25 July and 13 August 2015.

At the beginning of the dry-down treatment, all pots were overwatered and allowed to drain overnight; they were weighed in the following morning to obtain initial weight. Subsequently, all pots were weighed daily, and the difference between the weights on consecutive days was used to calculate daily transpiration losses for each pot. Well-watered pots received water daily to return their weight to 100 g less than the initial weight. To mimic the rate of development in soil water deficit in the field the drying pots were watered so that the water deficit developed slowly during approximately 2 weeks. Therefore, drying pots were watered if their net daily water loss was more than about 70 g so that daily net water loss was no greater than 70 g. In the first two experiments, soil drying was allowed to continue to impose severe stress after about 2 weeks to the point when TR of each drying plant decreased to less than or equal to 10% of the well-watered plants.

As it seemed possible that the stress level of the original two experiments would cause severe damage resulting in little or no TR and NF recovery, two additional experiments (Exp. 3 and 4) involving less severe soil drying were undertaken. In these two experiments, plants were dried only until TR reached 80% or 30% of the well-watered plants. Not all genotypes were tested in these less severe treatments. From the results of the Exp. 1 and 2, four genotypes were selected for the less severe drought treatments. The four genotypes included the two parent genotypes and two progeny genotypes: N05-UGA-848 (NF drought sensitive) and N05-UGA-886 (NF intermediate drought tolerance). In these two experiments, the two less severe treatments included only four replications. Exp. 3 occurred between 19 August and 13

September 2015, and Exp. 4 occurred between 29 September and 23 October 2015. Due to the comparatively short day lengths for Exp. 4, artificial lights were used to extend the day length to 14 h in this experiment; and if an occasional flower emerged, it was removed to maintain the plants in a vegetative state.

Nitrogen fixation measurement

The NF activity was measured daily in the greenhouse in which the plants were grown by introducing acetylene gas into the sealed pots by continuous flow and determining acetylene reduction activity (ARA). The protocol for determination of NF activity of the intact plants during a soil drying cycle was described by Devi and Sinclair (2013). Briefly, the pots were sealed so that the rhizosphere could be exposed to continuous flow of acetylene, which acts as a surrogate for atmospheric N₂ in the measurement of nodule activity.

Acetylene reduction activity was measured daily for all pots around noon. Ambient air was initially flowed through the sealed pots for 30 min. This was followed by flowing acetylene mixed with ambient air (1:9 v/v) at 1 L min⁻¹ through each pot. Acetylene is reduced in nodules to ethylene, and stable ethylene levels in the outflow from the pots were achieved in <15 min of exposure to the acetylene-air mixture. After this time, three individual gas samples were collected in syringes (1 mL) from the gas outflow of each pot, and ethylene concentration was measured using a flame ionization gas chromatography (Shimadzu Model GC-8A). Once the gas samples were collected, the pots were flushed with ambient air for 1 hour to remove any residual acetylene and ethylene.

Data processing

Transpiration rate data for each drying pot were analyzed by calculating for each day the ratio of its TR and the mean TR of the three well-watered pots of the same genotype. A second normalization was done for each drying pot, to minimize variability attributable to plant size in analyzing data for the five plants within a genotype. This second normalization was done by dividing the TR ratio of the drying pots on each day by the mean TR ratio of that same plant during the first 3 days of the experiment when the plant was still under well-watered conditions. This ratio was called the normalized transpiration ratio (NTR) and by definition was centered on 1.0 for each plant during the early phase of the experiments when water was still readily available in the soil.

Results of ARA during the soil drying phase were also normalized for each drought-stressed plant on each day using the same approach as used in the NTR calculation. The ARA ratio was calculated each day for each drying pot and then a second normalization, called normalized acetylene reduction activity (NARA) that was calculated by dividing the daily ratio by the mean

ARA value on the first 3 days of the experiment when the plant was still under well-watered condition.

Recovery

In the first two experiments, rewatering of the drying pots was done when the NTR reached a value of ≤ 0.1 . Following rewatering, TR and NF were measured for 5 days or more to observe the recovery capacity of each genotype. In Exp. 3 and 4, three treatments of soil water deficit were imposed by rewatering plants when NTR reached ≤ 0.8 , ≤ 0.3 , or ≤ 0.1 . For each genotype in Exp. 3 and 4, there were four replicate plants for each water-deficit treatment plus four well-watered pots.

A ratio was calculated for both TR and NF data for each rewatered pot on each day during recovery by dividing the observed values by the mean rate of the well-watered pots of the same genotype. Recovery normalized values (NTR and NARA) were calculated through a second normalization, by dividing the daily ratio for each rewatered pot by the mean ratio of TR and ARA of that pot measured on the last 3 days of the recovery phase.

Statistical analysis

The values of NTR and NARA during recovery from drought treatment were plotted against number of days following rewatering. Generally, the response to rewatering was described by two linear segments. In this case, the data were analyzed using a two-segment linear regression analysis (GraphPad Prism 6). The output of the regression gave the initial linear slope of the recovery and the time at which the plants had recovered to a plateau response, i.e., no further increase in activity. Comparisons of slopes and days to complete recovery among genotypes were done by using the 95% confidence interval generated for these variables in the linear regression analysis.

Results

Transpiration recovery

The recovery capacity of TR after soil dry-down to $NTR \leq 0.1$ in Exp. 1 and 2, in general, was fast as illustrated in [Figure 1A](#) and [1C](#) for genotypes N05-UGA-863 and N05-UGA-888. For all genotypes, recuperation of NTR to values equal to 1.0 or more occurred in less than 4 days for all genotypes ([Table 1](#)). Genotype N05-UGA-888 recovered TR faster than other genotypes, with recovered $NTR \geq 1.0$ in less than 2 days after rewatering. Hutcheson and PI 471938 showed slower recovery for transpiration in relation to the other genotypes studied, with recovery of TR after more than 3 d after rewatering.

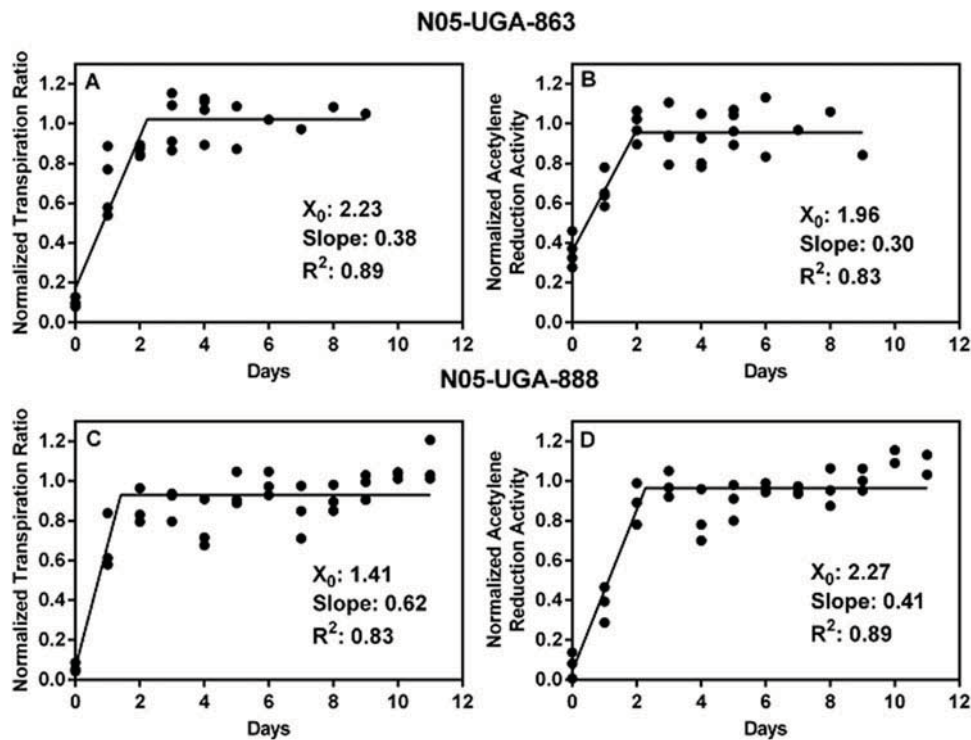


Figure 1. Recovery of normalized transpiration ratio (NTR) and normalized acetylene reduction activity (NARA) in two soybean genotypes: N05-UGA-863 (A and B) and N05-UGA-888 (C and D), submitted to dry-down until $NTR \leq 0.1$ plotted against days after re-watering. Slope refers to first-segment regression with an intercept at 1.0. X_0 refers to number of days taken to attain TR and NF recovery.

Table 1. Recovery capacity of normalized transpiration ratio (NTR) and normalized acetylene reduction activity (NARA) in 10 soybean genotypes, submitted to dry-down until $NTR \leq 0.1$ in Exp 1 and 2.

Genotypes	NTR slope	NTR threshold (days)	NARA slope	NARA threshold (days)
Hutcheson	0.26 c [†]	3.40 a [†]	0.18 d [†]	5.47 a [†]
PI 471938	0.27 c	3.16 ab	0.18 d	4.55 ab
N05-UGA-848	0.35 bc	2.62 bc	0.15 d	5.00 ab
N05-UGA-852	0.35 bc	2.67 bc	0.14 d	4.50 [‡]
N05-UGA-854	0.35 abc	2.74 abc	0.20 cd	3.99 b
N05-UGA-862	0.42 abc	2.59 abcd	0.29 bc	2.09 c
N05-UGA-863	0.39 abc	2.58 bc	0.59 a	1.34 d
N05-UGA-875	0.41 abc	2.18 cd	0.15 d	4.09 ab
N05-UGA-886	0.44 ab	2.10 cd	0.40 ab	2.08 c
N05-UGA-888	0.55 a	1.64 d	0.32 b	2.40 c

[†]Means followed by the same letter in columns do not differ from one another based on the 95 % confidence interval generated for each value by GraphPad Prism 6.

[‡]Result of a simple linear regression, value extrapolated at an intercept of 1.0.

In anticipation that the severe stress to $NTR \leq 0.1$ would result in major damage in the plant resulting in little recovery, Exp. 3 and 4 were conducted to observe recovery to the more modest stresses of $NTR \leq 0.8$ and ≤ 0.3 , in

Table 2. Recovery capacity of normalized transpiration ratio (NTR) in four soybean genotypes submitted to three different levels of drought stress in Exp. 3 and 4. Values within a genotype the followed by different letters indicate significant difference ($p < 0.05$) between treatments.

Genotypes	Dry-down levels	NTR Slope	Days until NTR \geq 1.0 after rewatering
Hutcheson	NTR \leq 0.8	0.30ab [†]	1.70b [†]
	NTR \leq 0.3	0.38a	2.15b
	NTR \leq 0.1	0.22b	3.85a
PI 471938	NTR \leq 0.8	0.15a	2.39a
	NTR \leq 0.3	0.31a	2.33a
	NTR \leq 0.1	0.24a	3.28a
N05-UGA-848	NTR \leq 0.8	0.06b	‡
	NTR \leq 0.3	0.34a	2.45a
	NTR \leq 0.1	0.47a	2.03a
N05-UGA-886	NTR \leq 0.8	0.17a	1.27b
	NTR \leq 0.3	0.29a	2.30a
	NTR \leq 0.1	0.31a	2.53a

[†]Means followed by the same letter in columns do not differ from one another based on the 95 % confidence interval generated for each value by GraphPad Prism 6.

[‡]Due to low slope for recovery, day to full recovery could not be calculated.

addition to the treatment of NTR \leq 0.1. The TR results obtained in Exp. 3 and 4 confirmed the rapid recovery capacity of TR after drought stress to NTR \leq 0.1 of genotypes Hutcheson, PI 471938, N05-UGA-848, and N05-UGA-886 (Table 2). Generally, recovery of NTR occurred in less than 3 d. The recovery of NTR from the two milder stress treatments was also rapid. An apparent exception was the NTR \leq 0.8 for N05-UGA-848, but in this case there was little initial decline in TR as a result of this mild stress treatment.

Nitrogen fixation recovery

Rapid recovery capacity for NF after dry-down to NTR \leq 0.1 was rapid as illustrated in Figure 1B and 1D. Although, the recovery of NF was generally slightly slower than that of TR. Across all results, the time to recovery of NTR ranged between 1.3 d for N05-UGA-863 to 5.5 d for Hutcheson (Table 1). In addition, genotypes N05-UGA-862, N05-UGA-886, and N05-UGA-888 also showed rapid recovery in slightly more than 2 d. Genotypes that showed recovery in about 5 d included PI 471938, N05-UGA-848, and N05-UGA-875. These results clearly indicated that the drought conditions imposed in these experiments did not result in permanent nodule damage causing a loss in an unrecoverable loss in nitrogen fixation activity.

The results from Exp. 3 and 4 also showed recovery in NF in less than 5 d for all treatments (Table 3). In four cases, the decrease in NF with drought treatment was so small that little recovery could be resolved. Modest water-deficit treatments that did not result in sufficient stress to detect recovery included NTR \leq 0.8 for N05-UGA-848 and N05-UGA-886 and NTR \leq 0.2 for Hutcheson.

Table 3. Recovery capacity of normalized acetylene reduction activity (NARA) in four soybean genotypes submitted to three different levels of drought stress in Exp. 3 and 4. Values within a genotype the followed by different letters indicate significant difference ($p < 0.05$) between treatments.

Genotypes	Dry-down levels	NARA slope	Days until NARA \geq 1.0 after rewatering
Hutcheson	NTR \leq 0.8	0.08	3.92a [†]
	NTR \leq 0.3	0.12	‡
	NTR \leq 0.1	0.11	‡
PI 471938	NTR \leq 0.8	0.06	4.84a
	NTR \leq 0.3	0.11	4.16a
	NTR \leq 0.1	0.14	4.98a
N05-UGA-848	NTR \leq 0.8	0.07	‡
	NTR \leq 0.3	0.19	2.00b
	NTR \leq 0.1	0.20	4.42a
N05-UGA-886	NTR \leq 0.8	0.01	‡
	NTR \leq 0.3	0.18	3.62a
	NTR \leq 0.1	0.36	2.05b

[†]Means followed by the same letter in columns do not differ from one another based on the 95 % confidence interval generated for each value by GraphPad Prism 6.

[‡]Due to low slope for recovery, day to full recovery could not be calculated.

Discussion

The first objective of this study was to document the rapidity of recovery of TR and NF upon watering drought-stressed plants. In fact, the recovery of both processes was fairly fast, although TR recovery tended to be slightly faster than NF. The range in recovery of NTR to well-watered rates was 1.6 to 2.4 d (Table 1) whereas the range for NF recovery was 1.3 to 5.5 d. However, there was no consistent pattern in rapid recovery of TR being associated with rapid recovery of NF.

In studies of Wang, Zhang, and Ding (2006), a Chinese soybean genotype was able to quickly recover TR and photosynthetic rate after 5 days of progressive drought stress and rewatering. The authors suggested that the plants had a strong osmotic adjustment ability that was retained after drought for a substantial span of time. However, these authors submitted the soybean plants only to a short period of stress (5 days), and did not consider the severity and duration of the stress. The nature of the water-deficit treatment may be important in resolving the nature of recovery as illustrated in the results from Exp. 3 and 4.

The results of these experiments indicated little or no permanent damage in soybean plants following a substantial stress to NTR \leq 0.1 that severely restricted rapid recovery of TR. Similarly, NF recovered fairly rapidly, indicating that no permanent damage was done in the nodules that restricted recovery in NF activity. The time-frame of NF recovery in these soybean plants is consistent with the results reported by King and Purcell (2005) and Mastrodomenico, Purcell, and King (2013).

The results of these experiments also offered some insight into genetic variation in recovery. Interestingly, the two parental genotypes, PI 471938 and Hutcheson, used in this study tended to have slower recoveries of both TR and NF than their progeny lines. The comparatively slow recovery of PI 471938 is somewhat surprising given its NF tolerance to soil drying (Devi and Sinclair 2013). However, the differences among genotypes in time of recovery of both TR and NF were only about 2 or 3 d and do not indicate that this is a response that may be a major limitation under field conditions.

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5 ARTIGO C - ESTRATÉGIAS PARA PROMOVER A NODULAÇÃO PRECOCE EM SOJA SOB SECA

Resumo

A fixação biológica de nitrogênio (FBN) é um processo sustentável que dispensa o uso suplementar de fertilizantes nitrogenados na cultura da soja [*Glycine max* (L.) Merrill]. Estratégias para estimular a nodulação precoce podem aumentar a eficiência da FBN sob condições estressantes como a seca. O objetivo desse trabalho foi avaliar os efeitos da inoculação de *Bradyrhizobium*, coinoculação de *Bradyrhizobium* e *Azospirillum*, e o uso de metabólitos secundários microbianos (MSM) nos parâmetros de nodulação e produtividade da soja em quatro experimentos de campo em duas épocas de cultivo, safras 2013/14 e 2014/15, na região sul do Brasil. Os tratamentos foram: controle não inoculado (Ni); Ni + fertilizante nitrogenado (100 kg ha⁻¹ na semeadura e 100 kg ha⁻¹ em pleno florescimento, na forma de ureia); Inoculado com *Bradyrhizobium* (I); Coinoculação com *Bradyrhizobium* + *Azospirillum brasilense* (Co-I); Co-I + metabólitos secundários microbianos (MSM) e I + MSM. Todos os ensaios foram realizados sem irrigação, sendo que o segundo ensaio, em 2013/14, foi severamente afetado pela seca e elevadas temperaturas. A coinoculação aumentou a nodulação nos estágios iniciais de desenvolvimento da cultura e resultou em maior crescimento da planta e acúmulo de N na parte aérea, especialmente em condições de seca. A adição de MSM ajudou a atenuar o efeito da seca sobre a nodulação e no primeiro ensaio aumentou a produção de grãos em 15% e 7% em relação ao tratamento com N mineral e somente inoculado com *Bradyrhizobium*, respectivamente. A estratégia de coinoculação estimula uma nodulação precoce e ajuda na manutenção da nodulação sob seca; além disso, a adição dos MSM traz benefícios para a nodulação e pode aumentar o rendimento de grãos.

Palavras-chave: Coinoculação, fatores Nod, fixação biológica de nitrogênio, LCO, rendimento de grãos.



Strategies to promote early nodulation in soybean under drought



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ABSTRACT

Biological nitrogen fixation (BNF) is a sustainable process that dismisses the use of supplemental N-fertilizers in soybean [*Glycine max* (L.) Merrill]. Strategies to provide early nodulation may increase the effectiveness of BNF under stressing conditions like drought. We assessed the effects of inoculation of *Bradyrhizobium*, co-inoculation of *Bradyrhizobium* and *Azospirillum*, and addition of microbial secondary metabolites (MSM) on nodulation parameters and soybean yield in four field experiments in two growing seasons, 2013/14 and 2014/15, in Southern Brazil. The treatments were: non-inoculated (Ni) control; Ni + N-fertilizer (100 kg ha⁻¹ at sowing and 100 kg ha⁻¹ at full flowering, as urea); Inoculated with *Bradyrhizobium* (I); Co-inoculated with *Bradyrhizobium* + *Azospirillum brasilense* (Co-I); Co-I + microbial secondary metabolites (MSM) and I + MSM. All trials were rainfed and the second trial in 2014/15 was severely affected by drought and high temperatures. The co-inoculation with *Azospirillum* increased the soybean nodulation at early developmental stages and resulted in higher shoot N accumulation and plant growth, especially under drought. The addition of MSM attenuated the effect of drought on nodulation and in one trial increased the grain yield by 15% and 7% in relation to the N-fertilizer and sole inoculation with *Bradyrhizobium*, respectively. The strategy of co-inoculation stimulates an early nodulation and helps the maintenance of nodulation under drought; moreover, the addition of MSM improves nodulation and may increase the grain yield.

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1. Introduction

Soybean [*Glycine max* (L.) Merrill] is the most widely grown oilseed worldwide, having reached a global 318.25 million metric ton in 2014/15 crop season (USDA, 2015). Currently, Brazil is the second largest producer, with 96.04 million metric ton in 2014/15 (CONAB, 2015). The success of the soybean crop in Brazil is in part attributed to the symbiosis with *Bradyrhizobium* and the biological nitrogen fixation (BNF) process, which provides nearly all N required and ensures high yields without supplemental mineral N (Alves et al., 2003; Hungria et al., 2006a).

However, drought and high temperatures (Hungria and Vargas, 2000; Sinclair et al., 2007; Chalk et al., 2010), or incompatibility of the microsymbiont with chemicals in the seed treatment (Hungria et al., 2015) may impair the BNF effectiveness. This biological process is negatively affected by drought even before the transpiration rate and photosynthesis (King and Purcell, 2005; Sinclair et al., 2007; Arrese-Igor et al., 2011). The impact of drought on plant and microsymbiont depends on the intensity, duration, and the plant developmental stage (Christophe et al., 2011). More drastic effects on nodulation and grain yield generally occur during the establishment of the symbiosis and formation of nodules (V2), full flowering (R2), or grain filling (R5) (Streeter, 2003; González-Dugo et al., 2010; Christophe et al., 2011).

Strategies to stimulate the early establishment of the symbiosis may result in increases in nodulation, nodule occupancy and more effective BNF, which might result in more even yields under drought (Hungria et al., 2006a; Chibeba et al., 2015). The co-inoculation of *Bradyrhizobium* with plant growth-promoting rhizobacteria like *Azospirillum* has been a beneficial strategy to promote early nodulation, BNF, and improve the crop's performance

Abbreviations: BNF, biological nitrogen fixation; CFU, colony forming units; DAE, days after emergence; LCO, lipo-chitoooligosaccharides; NDVI, normalized difference vegetation index; PET, potential evapotranspiration; MSM, microbial secondary metabolites.

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and grain yield (Hungria et al., 2013; Chibeba et al., 2015). These results are attributed to the ability of plant growth-promoting rhizobacteria to produce phytohormones that stimulate the root system for exploring the surrounding soil (Saharan and Nehra, 2011), and may increase tolerance to abiotic stresses like drought. Field experiments performed on Oxisols in different Brazilian regions have shown that co-inoculation with *Bradyrhizobium* and *Azospirillum* improves the soybean grain yield in relation to the sole inoculation with *Bradyrhizobium* or supplemental mineral N (Hungria et al., 2015).

Lipo-chitooligosaccharides (LCOs) are microbial secondary metabolites (MSM) essential for communication and establishment of the *Bradyrhizobium*-legume symbiosis (Cullimore et al., 2001; Gough, 2003). Although not acting directly on the growth and development of the host plant, MSM stimulate the symbiosis and promote the microbial growth, among other effects (Davies, 1992). Under greenhouse conditions, the use of MSM of rhizobia increased the nodulation of soybean by 21% in number and by 12% in mass of nodules, whereas under field conditions, MSM associated with rhizobial inoculant increased the number of nodules by 23.6% and the grain yield by 4.8% as compared with the sole inoculation with *Bradyrhizobium* (Marks et al., 2013).

There are few studies on the combination of these sustainable technologies with the inoculation of *Bradyrhizobium* in soybean limited by drought. The aim of this study was to evaluate the effect of co-inoculation of *Bradyrhizobium* with *Azospirillum* and addition of LCOs on traits related to BNF and grain yield of soybean under different hydric regimes in field experiments.

2. Material and methods

2.1. Experimental sites

Four field experiments were conducted in 2013/14 and 2014/15 cropping seasons. In 2013/14, two trials were installed in Londrina, PR, Brazil in a clay soil (23°11'S, 51°11'W, 620 m a.s.l., Cfa Köpen-Geiger climate, Rhodic Eutrudox soil type, USDA), sown at different dates. The first, 14 Oct. 2013 is considered early sowing, while the second, 23 Nov. 2013 is considered late sowing in the regular local sowing calendar (EMBRAPA, 2010). In 2014/15, the trials were repeated in Londrina (sown on 4 Nov. 2014), and in a different edafoclimatic condition in Ponta Grossa in a sandy-loam soil (25°13'S, 50°01'W, 880 m a.s.l., Cfb Köpen-Geiger climate type, Typic Hapludox soil type, USDA) sown on 18 Nov. 2014. Both soils have previously established and naturalized populations of *Bradyrhizobium*, since they have been cropped with soybean for more than 30 years.

Before sowing, topsoil samples (0–20 cm) were collected for chemical, granulometric, and microbiological analyses (Table 1). The soil rhizobial populations were estimated by the most probable number (MPN) technique (Vincent, 1970), using soybean cultivar BRS 360 RR as trap plant.

2.2. Experimental design and treatments

The experimental design was in randomized blocks with six replications, and the following treatments: non-inoculated (Ni) control; Ni+N-fertilizer (100 kg ha⁻¹ at sowing and 100 kg ha⁻¹ at full flowering, as urea); Inoculated with *Bradyrhizobium* (I); Co-inoculated with *Bradyrhizobium*+*Azospirillum brasilense* (Co-I); Co-I+Microbial secondary metabolites (MSM); I+MSM. In 2013/14, the soybean cultivar was BMX Potência RR; in 2014/15, the cultivar was BRS 359 RR in both sites. Plots consisted of eight lines of 6.0 m in length spaced 0.50 m apart and density of 280,000 plants ha⁻¹.

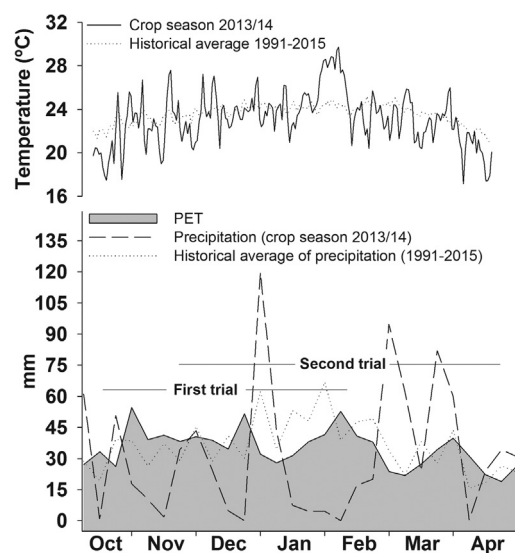


Fig. 1. Daily average temperature (°C), potential evapotranspiration (PET), precipitation (mm) every 7 days during the crop season period (2013/14), and historical (1991–2015) average temperature and precipitation in Londrina. Data on historical averages were adapted from Sibaldelli and Farias (2015).

The *Bradyrhizobium* inoculants contained the strains SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7) of *B. japonicum* and *B. diazoefficiens*, respectively (with 1.2×10^9 colony forming units (CFU) mL⁻¹). The dose of inoculant was calculated to deliver 1.2×10^6 cells per seed. Treatments with co-inoculation received *Azospirillum brasilense* strains Ab-V5 and Ab-V6 (1×10^8 CFU mL⁻¹) in a dose to deliver 1.2×10^5 cells per seed. Treatments with MSM received the LCOs at 100 mL 50 kg⁻¹ seeds, obtained as described in Marks et al. (2015).

2.3. Field management

Plants received 250 or 300 kg ha⁻¹ of N–P–K (0–20–20) in furrow, simultaneously the sowing in both trials, in 2013/14 and 2014/15, respectively. No N-fertilizer was applied, except for the N-fertilized control plots. Seeds were not treated with fungicides or insecticides. At V4 stage (Fehr and Caviness, 1977), plants were leaf-sprayed with 20 g ha⁻¹ of Mo (Na₂MoO₄·2H₂O) and 2.5 g ha⁻¹ of Co (CoCl₂·6H₂O).

The potential evapotranspiration (PET), water balance for Londrina (calculated by Pan Evaporation Method, based on the average daily values for 2013/2014) and the mean daily temperatures and rainfall during the 2013/14 crop season trials, in addition to the historical average for rainfall and air temperature, are shown (Fig. 1). During the crop cycle in 2014/15, the mean temperature ranged between 19.1–29.0 °C and 15.1–27.5 °C, and the rainfall was 718 mm and 889 mm in Londrina and Ponta Grossa, respectively.

2.4. Plant sampling, analysis, and grain yield

The dynamics of nodulation were evaluated in the trials performed in 2013/14 crop season. Five plants were collected on the second and on the seventh line of the plot, i.e., 2 or 3 plants each side, within 0.5 m in the line, and the next sampling performed after 0.4 m forward to avoid effects of compensatory growth caused by gaps resulting from the previous sampling. Samples were taken at 10, 15, 25, 45, 65, and 95 days after emergence (DAE) for the first trial and at 12, 18, 27, 40, 70, and 115 DAE for the second trial, corresponding to V1, V2, V4, R2, R4-5, and R6-7 growth stages (Fehr and Caviness, 1977). The shoots were separated from roots, and dried at 65 °C for 72 h for assessment of shoot and root dry weights. The

Table 1
Chemical characteristics, granulometric fractions, and population of *Bradyrhizobium* at the 0–20 cm topsoil layer in the experimental field sites before sowing.

Site	Crop season	pH	cmol _c dm ⁻³			mg dm ⁻³		N-NH ₄ ⁺ mg kg ⁻¹	N-NO ₃ ⁻ mg kg ⁻¹	Total-N g kg ⁻¹	C g dm ⁻³	Clay g kg ⁻¹	Silt	Sand	<i>Bradyrhizobium</i> cells MPN g ⁻¹
			Al+H	K	Ca+Mg	P									
Londrina	2013/14	5.7	3.83	0.95	7.55	22.0	11.61	2.38	1.57	14.01	753	166	80.5	2.4 × 10 ⁵	
Londrina	2014/15	5.4	3.53	0.61	6.40	20.0	9.26	4.33	1.55	12.70	238	30.0	732	3.5 × 10 ⁴	
Ponta Grossa	2014/15	5.1	3.89	0.45	4.42	15.0	9.26	4.33	1.55	18.70	238	30.0	732	2.9 × 10 ⁴	

pH (CaCl₂); P (Mehlich I); H + Al (SMP); Al, Ca, Mg, K (KCl). MPN = Most Probable Number.

nodules from the root crown were counted and weighed for assessment of the number and dry weight of nodules. Shoots were ground (<10 mesh) and total shoot N content was determined in sulfuric extracts by the green salicylate colorimetric method (Searle, 1984).

Physiological parameters were measured with a portable gas-exchange meter (LCpro-SD, ADC BioScientific Ltd., UK) during the full flowering of plants (R2) for the two trials in 2013/14 crop season. Determinations included stomatal conductance, net photosynthetic rate, transpiration rate and intercellular CO₂ concentration. SPAD units were determined in the recently expanded trifoliolates with a portable meter, model SPAD-502 (Konica Minolta Sensing Inc., Osaka, Japan), and converted into leaf-chlorophyll concentration (μg cm⁻²) (Kaschuk et al., 2010). These analyses were performed in the same trifoliolates, in two plants per plot, in the morning (9–11 a.m.). The NDVI (normalized difference vegetation index) was measured using a 505 Handheld device GreenSeeker[®] sensor (Ntech Industries, Inc., USA) in the same day. The NDVI values range from -1 to 1 so that the closer to 1 the greener is the vegetation.

Grain yield at maturation (R8) was determined by harvesting a central area of each plot (8 m²). Harvests occurred on 26 Feb. 2014 and on 23 Apr. 2014, for the first and second trial, respectively. Cleaned seeds were weighed and the values corrected to 13% of moisture, after determination of humidity in a grain moisture tester (Gehaka Agri model G800, São Paulo, Brazil).

In the 2014/15 crop season, plants were sampled only at 35 DAE in Londrina and at 54 DAE in Ponta Grossa for assessments of nodulation (number and dry weight), and for analysis total shoot N content. At R8 stage (12 Mar. 2015 in Londrina, and 31 Mar. 2015 in Ponta Grossa), plants were harvested for analysis of grain yields, as previously described.

2.5. Statistical analyses

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

3. Results

The physiological responses of plants measured at the second trial in 2013/14 confirmed the stressing conditions caused by drought associated to high temperatures. There was reduction in gas exchanges, photosynthesis (-32%) and transpiration rates (-23%), stomatal conductance (-50%) and intercellular CO₂ (-10%) compared with the first trial, in the average of all treatments. The treatments had only minor effects on the physiological measurements (Table 2). In the first trial, the non-inoculated plants showed higher net photosynthesis (17%), transpiration rate (8%), stomatal conductance (40%), and intercellular CO₂ (8%) in relation to the other treatments. In the second trial, there was significant difference only for stomatal conductance, which was 26% higher in plants inoculated with *Bradyrhizobium*; and chlorophyll content, 7%

higher in plants inoculated with *Bradyrhizobium* + MSM compared with the non-inoculated plants.

3.1. Nodulation in 2013/14 crop season

In the first trial, the control plants that received mineral N showed a consistent reduction in number and dry weight of nodules, in all samplings (Fig. 2A and 2C). There was more number of nodules in plants co-inoculated with *Bradyrhizobium* + *Azospirillum*, than the non-inoculated plants at 10 (33%) and 65 DAE (38%); compared with plants that received N fertilization, nodulation was higher (78% in mass and 67% in number) at 10 DAE. At 25 DAE, plants inoculated with *Bradyrhizobium* + MSM showed 30% more nodules than plants only inoculated with *Bradyrhizobium* and twice as more than plants that received mineral N. The total shoot N content in plants showed similar trend as shoot dry weight (not shown), wherein plants with mineral N showed higher initial N concentration in leaves in most of the sampling dates, followed by a sharp decrease at 90 DAE (Fig. 2E).

For the second trial, there was a general decrease in nodulation (-52% in mass, -31% in number) compared with the first trial (Fig. 2B and 2D), but the negative effect of mineral N on nodulation was less evident. At 12 DAE, plants inoculated with *Bradyrhizobium* and *Bradyrhizobium* + MSM had 20% more mass and 31% more number of nodules than the non-inoculated plants. At 40 and 115 DAE plants inoculated with *Bradyrhizobium* + MSM stood out for number of nodules, showing 28% and 21% more than plants inoculated only with *Bradyrhizobium*, respectively. However, higher dry weight of nodules (37%) at 40 DAE was observed in plants co-inoculated with *Azospirillum*. Considering the total shoot N at 27 DAE (Fig. 2F), the plants co-inoculated with *Azospirillum* accumulated 97% more N than the N-fertilized plants. At 18 and 40 DAE, non-inoculated plants and inoculated with *Bradyrhizobium* stood out for total shoot N. At 70 DAE higher values were observed in non-inoculated and N-fertilized plants, but both followed a decrease at 115 DAE, when the plants inoculated with *Bradyrhizobium* and co-inoculated with *Azospirillum* accumulated, respectively, 38% and 21% more N than N-fertilized plants (Fig. 2F).

3.2. Nodulation in 2014/15 crop season

The application of N-fertilizer reduced the number and dry weight of nodules in both trials (Table 3), by -35% and -42%, respectively, in Londrina, and by -28% and -19% in Ponta Grossa, compared with the average of the other treatments. The total shoot N content in plants grown in Londrina was 20% higher when inoculated only with *Bradyrhizobium* compared with plants of treatments non-inoculated, co-inoculated + MSM and inoculated with *Bradyrhizobium* + MSM. In Ponta Grossa, there was 30% more total shoot N content in plants with mineral N, compared with plants non-inoculated and co-inoculated with *Azospirillum* + MSM (Table 3).

Table 2

Net photosynthetic and transpiration rates, stomatal conductance, intercellular CO₂ concentration, NDVI (normalized difference vegetation index), and chlorophyll content in soybean analyzed in full flowering (R2) period of plants in two trials in Londrina in 2013/14 crop season.

Treatments	Photosynthetic rate μmol CO ₂ m ⁻² s ⁻¹	Transpiration rate mmol H ₂ O m ⁻² s ⁻¹	Stomatal conductance mol H ₂ O m ⁻² s ⁻¹	Intercellular CO ₂ μmol CO ₂ mol ⁻¹	NDVI	Chlorophyll μg cm ⁻²
	First trial					
Non-inoculated	15.78 a	3.85 a	0.340 a	227.9 a	0.878 a	19.68 a
Non-inoculated + N-fertilizer (200 kg ha ⁻¹) ^a	13.32 b	3.61 a	0.217 b	211.9 abc	0.889 a	20.04 a
Inoculated with <i>Bradyrhizobium</i>	12.59 b	3.38 a	0.210 b	213.6 abc	0.877 a	19.56 a
Co-inoculation with <i>Bradyrhizobium</i> + <i>Azospirillum</i>	13.43 b	3.83 a	0.186 b	202.9 bc	0.881 a	20.51 a
Co-inoculation with <i>Bradyrhizo-</i> <i>bium</i> + <i>Azospirillum</i> + MSM	12.35 b	3.49 a	0.212 b	219.0 ab	0.878 a	20.15 a
Inoculated with <i>Bradyrhizobium</i> + MSM	13.80 b	3.33 a	0.191 b	200.2 c	0.881 a	20.63 a
p-value	<0.01	0.0305	<0.01	<0.01	0.4869	0.2001
CV (%)	7.21	8.82	20.51	4.48	1.27	4.16
	Second trial					
Non-inoculated	9.43 a	2.62 a	0.106 b	184.3 a	0.645 a	19.46 b
Non-inoculated + N-fertilizer (200 kg ha ⁻¹)	9.67 a	2.77 a	0.106 b	192.8 a	0.658 a	20.04 ab
Inoculated with <i>Bradyrhizobium</i>	10.80 a	2.80 a	0.138 a	196.4 a	0.610 a	19.56 ab
Co-inoculation with <i>Bradyrhizobium</i> + <i>Azospirillum</i>	9.02 a	2.79 a	0.114 ab	193.0 a	0.632 a	20.51 ab
Co-inoculation with <i>Bradyrhizo-</i> <i>bium</i> + <i>Azospirillum</i> + MSM	9.84 a	2.78 a	0.099 b	200.4 a	0.643 a	19.71 ab
Inoculated with <i>Bradyrhizobium</i> + MSM	9.40 a	2.50 a	0.087 b	187.9 a	0.638 a	20.85 a
p-value	0.0652	0.6661	<0.01	0.1143	0.0688	0.0269
CV (%)	9.88	13.66	14.40	5.19	4.02	3.88

MSM – Microbial secondary metabolites; CV – Coefficient of variation; ^aN-fertilizer (100 kg ha⁻¹ at sowing and 100 kg ha⁻¹ at full flowering, as urea); Means (n = 6) followed by the same letters in the same column do not differ one another (Tukey, P ≤ 0.05).

Table 3

Number of nodules, nodule dry weight, total shoot N content, at 35 days after emergence (DAE) in Londrina and at 54 DAE in Ponta Grossa, in soybean plants (BRS 359 RR) cultivated in 2014/15 crop season.

Treatments	Londrina			Ponta Grossa		
	Number of nodules (n° plant ⁻¹)	Nodule dry weight (mg plant ⁻¹)	Total shoot N content (mg N plant ⁻¹)	Number of nodules (n° plant ⁻¹)	Nodule dry weight (mg plant ⁻¹)	Total shoot N content (mg N plant ⁻¹)
Non-inoculated	21.4 a	45.70 a	138.8 b	115 ab	430.1 ab	206.5 b
Non-inoculated + N-fertilizer (200 kg ha ⁻¹) ^a	12.4 b	17.15 b	163.4 ab	86.0 b	356.2 b	299.5 a
Inoculated with <i>Bradyrhizobium</i>	17.5 a	40.22 a	174.5 a	108 ab	392.1 ab	259.7 ab
Co-inoculation with <i>Bradyrho-</i> <i>bium</i> + <i>Azospirillum</i>	18.4 a	43.34 a	155.9 ab	114 ab	425.3 ab	248.9 ab
Co-inoculation with <i>Bradyrho-</i> <i>bium</i> + <i>Azospirillum</i> + MSM	19.0 a	33.76 a	141.7 b	135 a	496.4 a	209.3 b
Inoculated with <i>Bradyrhizobium</i> + MSM	19.5 a	39.99 a	141.6 b	123 ab	460.1 ab	277.2 ab
p-value	<0.01	<0.01	<0.01	0.017	0.0423	<0.01
CV (%)	15.04	19.07	9.83	19.00	17.13	16.94

MSM – Microbial secondary metabolites; CV – Coefficient of variation. ^aN-fertilizer (100 kg ha⁻¹ at sowing and 100 kg ha⁻¹ at full flowering, as urea); Means (n = 6) followed by the same letters in the same column do not differ one another (Tukey, p ≤ 0.05).

Table 4

Soybean grain yield in trials carried out in Londrina during 2013/14, and in Londrina and Ponta Grossa during 2014/15.

Treatments	Trials			
	1 – Londrina (2013/14)	2 – Londrina (2013/14)	3 – Londrina (2014/15)	4 – Ponta Grossa (2014/15)
	(kg ha ⁻¹)			
Non-inoculated	3505 a	1322 ab	3052 bc	2825 b
Non-inoculated + N-fertilizer (200 kg ha ⁻¹) ^a	3453 a	1454 a	3126 bc	3126 a
I (Inoculated with <i>Bradyrhizobium</i>)	3433 a	1119 abc	3360 ab	2927 ab
I + Azo (co-inoculation with <i>Bradyrhizobium</i> and <i>Azospirillum</i>)	3364 a	768 c	2930 c	2830 b
I + Azo + MSM (microbial secondary metabolites)	3393 a	991 abc	2937 c	2878 b
I + MSM	3433 a	888 bc	3596 a	3003 ab
p-value	0.9457	<0.01	<0.01	<0.01
CV (%)	7.25	23.9	5.73	4.58

MSM – Microbial secondary metabolites; CV – Coefficient of variation. ^aN-fertilizer (100 kg ha⁻¹ at sowing and 100 kg ha⁻¹ at full flowering, as urea); Means (n = 6) followed by the same letters in the same column do not differ one another (Tukey, p ≤ 0.05).

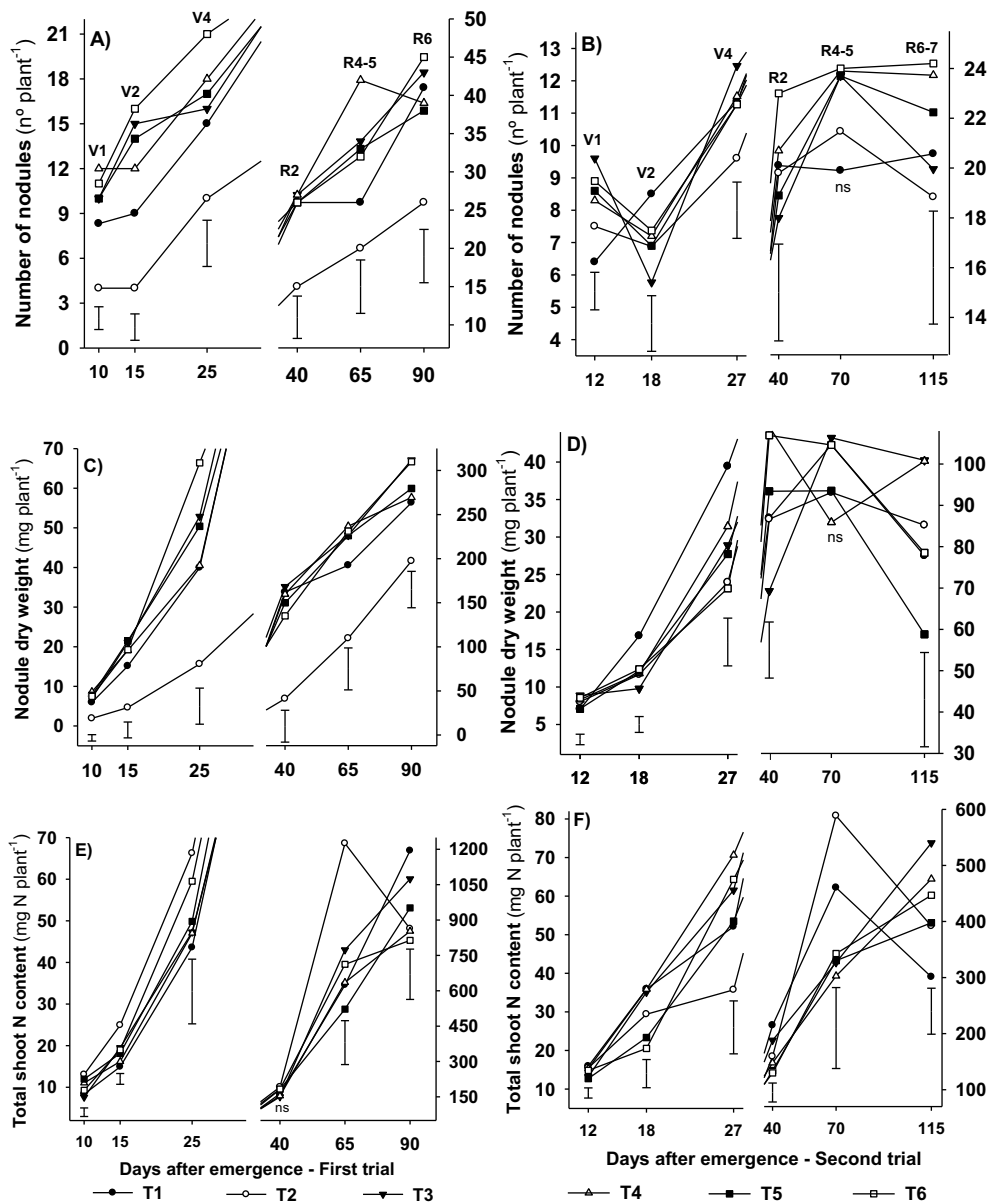


Fig. 2. Number of nodules (A and B); nodule dry weight (C and D) in root crown in different days after emergence (DAE), and total shoot N content (E and F) in soybean plants in trials performed in Londrina in crop 2013/14: first trial (Oct/2013–Feb/2014) and second trial (Nov/2013–Apr/2014). —●— T1 – non-inoculated (Ni) control; —○— T2 – Ni+N-fertilizer (100 kg ha⁻¹ at sowing and 100 kg ha⁻¹ at full flowering, as urea); —▼— T3 – Inoculated with *Bradyrhizobium* (I); —▲— T4–Co-inoculation with *Bradyrhizobium* spp. + *Azospirillum brasilense* (Co-I); —■— T5–Co-I + Microbial secondary metabolites (MSM); —□— T6–I + MSM. Vertical bars represent the standard deviation (SD) for Tukey's test ($P \leq 0.05$) ($n = 6$).

3.3. Grain yield

In 2013/14, there was no effect of treatments on grain yield in the first trial, which averaged 3430 kg ha⁻¹ (Table 4). In the second trial, the grain yield dropped severely as result of drought during the initial developmental stage and at full flowering, and averaged 1090 kg ha⁻¹, 68% less than the average of first trial (Table 4). In this case, plants that received mineral N had higher yield, but not differing from plants non-inoculated, inoculated with *Bradyrhizobium*, and co-inoculated with *Azospirillum* + MSM.

In 2014/2015, plants inoculated with *Bradyrhizobium* + MSM showed the highest yield in Londrina, but did not differ from plants inoculated only with *Bradyrhizobium*. In Ponta Grossa, the N-fertilized plants increased the grain yield by 10% compared with non-inoculated plants, co-inoculated with *Azospirillum* and co-inoculated + MSM (Table 4).

4. Discussion

The use of biofertilizers and biomolecules in the soybean production system helps to reduce costs and minimize environmental impacts caused by mineral N-fertilizers. Biofertilizers benefit plants by means of BNF (Alves et al., 2003; Hungria et al., 2006a), production of phytohormones (Saharan and Nehra, 2011), phosphate solubilization (Saikia et al., 2010), and induction of plant resistance to biotic and abiotic stresses (Gurska et al., 2009). Studies have confirmed the effectiveness of co-inoculation with *Bradyrhizobium* and *Azospirillum* in increasing soybean grain yield (Hungria et al., 2013, 2015) and stimulating earlier nodulation (Chibeba et al., 2015), and also positive effects of MSM on soybean and corn yields (Marks et al., 2013, 2015).

In the present work, there was a clear influence of the climate on the performance of soybean and on attributes related to BNF,

when a dry period and high air temperatures that reached 36.6 °C occurred in the 2013/14 crop season and impaired mainly the second trial. During this season, the air temperature was far above the historical averages, whereas the rainfall was lower during some critical stages of the second trial, i.e., just after the sowing (V2) and during the flowering (R1–R3). Water stress in the second trial was confirmed by physiological data like photosynthesis and transpiration rates, which decreased in comparison with the first trial. According to Flexas et al. (2004), the stress level can be quantified by the stomatal conductance, wherein values $\geq 0.2 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ represents well watered plants; between $0.1\text{--}0.2 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ moderate drought stress, and $\leq 0.1 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ severe drought stress. Considering this classification, the plants in the second trial were under moderate drought stress at R2 stage, what is corroborated by lower NDVI indices. This environment also resulted in a strong decrease in nodulation and yield.

The soybean crop reaches the maximum hydric needs between flowering and grain filling, 7–8 mm day⁻¹ (EMBRAPA, 2010). In studies performed in Londrina along 15 cropping seasons, the maximum grain yield was obtained with 650–700 mm of water sheet, well distributed throughout the crop cycle (Farias et al., 2010). In our study during the 2013/14 the crop season, the total rainfall was 496 mm in the first trial, and 695 mm in the second. However, there was an uneven distribution during the second trial, with only 18 mm in 20 days during V2–V4, a critical period of plant development, when the symbiosis is being established. Water restriction persisted in the R1–R5 (early-flowering and beginning of grain formation), with only 30 mm accumulated in 40 days. In this period, the plants of the first trial were at advanced reproductive stages (R6–R7), and were not as severely affected by drought as the plants in the second trial.

The nodulation in the first trial of 2013/14 increased at initial phases in plants co-inoculated with *Azospirillum*, which is critical for an early establishment of the symbiosis (Chibeba et al., 2015). There was also a positive effect of the MSM, as previously observed (Marks et al., 2013). In the second trial, the early stages of plant development and the establishment of the symbiosis occurred under dry conditions that severely decreased the nodulation in comparison with the first trial, leading to a consistently lower nodulation along all over the crop cycle. Even so, the inoculation with *Bradyrhizobium*, either as sole inoculation, co-inoculated with *Azospirillum*, or added with MSM, provided earlier nodulation. However, co-inoculation with *Azospirillum* stimulated more the mass of nodules, whereas the MSM stimulated the number. Such differences seem to be related to the mechanisms of actions, in which *Azospirillum* mainly act by hormonal effect, increasing the nodule size and number (Chibeba et al., 2015; Hungria et al., 2013), whereas MSM intermediate the signaling between *Bradyrhizobium* and the host plant via LCOs, increasing the number of nodules (Cullimore et al., 2001; Marks et al., 2013). The stimulation of nodulation by MSM confirms its key role in the formation and establishment of *Bradyrhizobium*-Legumes symbiosis, as observed in *Medicago* spp. (Cullimore et al., 2001).

The co-inoculation provided more accumulation of N in shoots at the early stages of plant development. *Azospirillum* is known for improving plant traits that may help to cope with water deficit, like branching of roots and increased density of root hairs (Cassán and Garcia De Salomone, 2008; Hungria et al., 2015). Consequently, there are also benefits to the BNF and plant growth. Chibeba et al. (2015) observed that the beneficial effects of *Bradyrhizobium*-*Azospirillum* co-inoculation were more evident in the field than in greenhouse, suggesting that *Azospirillum* may help plants to overcome environmental stresses in the field. Hungria et al. (2015) also confirmed this effect, wherein *Azospirillum*-*Bradyrhizobium* co-inoculation increased the grain yield of soybean under moderate water restriction.

The addition of N-fertilizer in our study decreased the nodulation when the availability of water was not restrictive, corroborating Hungria et al. (2006b) wherein the application of 200 kg ha⁻¹ of N-fertilizer reduced the nodulation and the benefits of BNF, and did not increase yields. The non-inoculated plants, on the other hand, showed a similar or even higher nodulation than the inoculated treatments. This is because all experimental areas have been cropped with soybean for more than 30 years and have a high population of pre-established bradyrhizobia in the soil, as revealed the analysis for bradyrhizobial population. However, although the population established is able to colonize and promote nodulation, they might not be as effective as the strains just added via inoculants. Moreover, the nodulation process is usually delayed when relying only on the rhizobial population established in the soil, which may affect the plant performance and grain yield. In the case of rainfed crops, an early establishment of the symbiosis is very important if any restrictive factor like drought occurs thereafter. In fact, inoculation in areas with established bradyrhizobial population have shown an average increase in yields of about 8% (Hungria et al., 2013).

The negative effect of water restriction on soybean depends on its phenological stage (Avila et al., 2013; Ku et al., 2013). Drought severity on grain yield may be alleviated when it occurs at early phases, because plants would have time to recover, but occurrence at reproductive stages may lead to deeper drops in yields, depending on the intensity (Chalk et al., 2010). Soybean plants assimilate about 20% of total N until early flowering stage (R1 stage), and 80% of N during the reproductive stage after R2 (Ohyama et al., 2013). Therefore, the continuous assimilation of nitrogen via BNF during the vegetative stage and after initial reproductive stage is essential for plant performance and seed yield (Ohyama et al., 2013).

In the second trial of 2013/14, the occurrence of drought at two critical stages of soybean development (V2–V4 and R1–R5) affected the BNF process, with reduction of N accumulation, and expressive reduction of yield by 68% less than the first trial. In this case, the treatment with mineral N showed some gain in yield over two inoculated treatments. As BNF is vulnerable to drought (Sinclair et al., 2007; Arrese-Igor et al., 2011; Cerezini et al., 2014), some studies have shown that supplemental mineral N could increase grain yield under drought (Purcell and King, 1996; Ray et al., 2006; Salvagiotti et al., 2008). However, this gain did not compensate the costs of using N fertilizer (Hungria et al., 2006b), including environmental costs associated to N production and losses. Even 'starter' doses of mineral N have shown no significant effect on grain yield (Alves et al., 2003), and may even impair the establishment of the symbiosis and its capacity for N supply at later stages like pod filling. Even with a gain in yield under drought, N-fertilizer was not economically viable (Hungria et al., 2013; Salvagiotti et al., 2008). Considering the most contrasting yield gain of 686 kg ha⁻¹ with N-fertilizer, the approximate soybean (US\$ 0.25 per kg) and urea (US\$ 1.00 per kg of N) prices in the Brazilian market, the N-application was not profitable.

Despite positive effects on nodulation, inoculation, co-inoculation, or use of MSM not necessarily resulted in higher yields, which increased only in the second growing season in Londrina. However, there were clear effects in terms of N accumulated in shoot biomass. In the first trial of 2013/14, when the climate conditions (temperature and rainfall) were less severe, the N-fertilized plants accumulated more N in the shoots. However, as the sink strength increased with the pod filling, the accumulated N in N-fertilized plants decreased sharply, whereas the BNF-based plants kept the accumulated N levels, probably because plants that received N mineral entered in senescence earlier than plants inoculated with *Bradyrhizobium*. Under the severe weather conditions observed in the second trial of 2013/14 growing season, both N-fertilized and BNF-based on soil bradyrhizobial population stood

out in terms of N accumulation in shoots at early reproductive developmental stages. However, both decreased sharply at the pod filling stage, whereas the inoculated plants kept higher N contents in the biomass that could have supported higher grain yields if mild climate conditions had occurred thereafter. Despite higher accumulation of N in N-fertilized plants at vegetative and early reproductive stages, there was a drop in accumulated N at pod filling, probably because the decreased nodulation by N-fertilizer was not enough to supply the demanded N at later stages (Kaschuk et al., 2016). Under a more severe climatic condition as in the second trial, both N-supplied and BNF-based on the soil Bradyrhizobial population led to a drop in N accumulation in plant shoots at pod filling stages. Even sometimes inoculation does not result in higher grain yields, it is the most sustainable way to supply N for soybeans and assure that among several factors comprising the yield components, at least N be as less restrictive as possible (Alves et al., 2003; Chalk et al., 2010). In addition, the N remaining in the crop residues can be an important source of N for the subsequent crop, increasing the sustainability of the production system.

In conclusion, severe drought associated to high temperatures sharply decrease nodulation and yield of soybean. Even under such condition, supplemental mineral N does not result in an economically viable increase of grain yield. On the other hand, estimates show that the soybean crop in Brazil saves about US\$ 7 billion year⁻¹ with the BNF process (Hungria et al., 2013). The strategy of inoculation, co-inoculation or use of MSM, even in soils with established rhizobial population, increase the nodulation at initial stages and help its maintenance under drought. These practices assure higher N accumulation at later reproductive stages and may eventually increase grain yield.

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6 CONSIDERAÇÕES FINAIS

Apesar de a soja ser uma das leguminosas mais bem estudadas no Brasil, o agronegócio da cultura tem sofrido perdas consideráveis causadas por restrição hídrica, em que a fixação biológica do nitrogênio é um dos processos mais prejudicados. Com isso, o estudo e aplicação de estratégias que promovam benefícios à fixação biológica de nitrogênio e estabilidade produtiva de soja sob seca poderão favorecer o agronegócio dessa cultura frente às adversidades climáticas.

As diferenças genótípicas em soja quanto à capacidade de tolerar e manter os componentes de produção após um período de seca foram confirmadas no artigo A; entretanto, os mecanismos de proteção/tolerância que cada genótipo utiliza, para manutenção do seu metabolismo no período de restrição hídrica, não são os mesmos, sendo que uma série de genes e mecanismos fisiológicos podem ser ativados, de forma diferente, com intuito de proteger a planta e a fixação de N₂ durante o período adverso. Durante a restrição hídrica, genótipos tolerantes têm habilidade de manter a fotossíntese, promover a alongação do sistema radicular, manter a atividade da enzima glutamina sintetase e promover menor acúmulo de ureídeos nas folhas. Essas são características desejáveis que contribuem para o aumento da tolerância da fixação biológica de nitrogênio à seca. Destaca-se ainda que os genótipos BRB-27, R02-1325 e BRB-26 mantêm a capacidade produtiva de grãos após restrição hídrica e reidratação, sendo fontes genéticas potenciais para o estudo e desenvolvimento de cultivares de soja tolerantes à seca.

Diferenças genótípicas em soja quanto à capacidade de tolerar e apresentar recuperação após um período de seca severa também foi constatado no artigo B, em relação à capacidade transpiratória e atividade da nitrogenase estimada pelo método não destrutivo da redução de acetileno. A recuperação desses parâmetros foi observada nos dez genótipos de soja avaliados, demonstrando que os efeitos da seca severa foram reparáveis, pois nenhum dano permanente ocorreu nas plantas analisadas. Além disso, foi possível concluir, com esse estudo, que a recuperação da transpiração e da atividade da nitrogenase após reidratação das plantas é rápida; no entanto, as taxas transpiratórias são restauradas mais rapidamente que a atividade de fixação do N₂ após reidratação da planta.

Em relação à simbiose soja-*Bradyrhizobium*, estratégias avaliadas no artigo C apontam o potencial da coinoculação de *Bradyrhizobium* com

Azospirillum, como previamente demonstrado por Chibeba et al. (2013) e Hungria, Nogueira e Araújo (2015). A interação positiva entre essas bactérias vão além do estímulo à nodulação e fixação biológica de nitrogênio, pois fatores indiretos contribuem para aumentar o potencial produtivo da cultura, como a promoção do crescimento vegetal e aumento da tolerância da planta a situações de estresses, como a seca; característica importante em plantas dependentes da fixação de N₂, uma vez que é bem documentado que períodos de seca e elevadas temperaturas são os fatores abióticos mais limitantes para a eficácia desse processo biológico (SERRAJ; SINCLAIR; PURCELL, 1999; HUNGRIA; KASCHUK, 2013; CERZINI et al., 2014). Constatou-se ainda, que a adição de metabólitos secundários microbianos, os oligossacarídeos lipoquitínicos, proporcionam vantagens na nodulação e performance da planta, o que pode aumentar o rendimento de grãos de soja.

Finalmente, conclui-se que a seleção e utilização de genótipos de soja tolerantes à seca, juntamente com práticas sustentáveis que contribuam para favorecer a nodulação em soja, como a coinoculação de *Bradyrhizobium* com *Azospirillum* e uso de metabólitos secundários microbianos, são potenciais ferramentas para aumento da estabilidade produtiva da cultura da soja em períodos de restrição hídrica.