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MARIA DOLORES PISSOLATO

ALLEVIATING THE EFFECTS OF WATER DEFICIT ON SUGARCANE THROUGH NITROGEN NUTRITION

AMENIZANDO OS EFEITOS DO DÉFICIT HÍDRICO EM CANA-DE-AÇÚCAR COM A NUTRIÇÃO NITROGENADA

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RESUMO

A água e o nitrogênio (N) são alguns dos fatores mais limitantes na produção agrícola mundial, ambos fortemente associados à assimilação fotossintética do CO_2. As modificações no metabolismo de N em condição de déficit hídrico são relativamente pouco estudadas em plantas C4 e as informações ainda se encontram fragmentadas. Sabe-se que o radical óxido nítrico (NO) é uma importante molécula sinalizadora em vários sistemas biológicos, e tem papel protetor agindo principalmente no combate aos radicais livres formados sob estresse oxidativo. Os mecanismos de síntese do NO e sua regulação são complexos e pouco compreendidos. No entanto, a nitrato redutase (NR) é uma enzima chave para a assimilação de nitrogênio nas plantas e tem sido proposta como uma importante via enzimática para a produção de NO a partir da redução do nitrito. Diante do exposto, o objetivo deste trabalho foi avaliar a influência da nutrição nitrogenada nas respostas fisiológicas de cana-de-açúcar ao déficit hídrico, considerando a atividade fotossintética, o metabolismo antioxidante e o crescimento das plantas. A hipótese a ser testada é de que plantas que recebem mais nitrato como fonte de N terão uma maior atividade da NR e em situação de déficit hídrico produzirão mais NO, quando comparadas as que recebem menos nitrato. Espera-se que o NO produzido desempenhe um papel atenuador do déficit hídrico em plantas de cana-de-açúcar, reduzindo o dano oxidativo e melhorando a fotossíntese e o crescimento das plantas sob baixa disponibilidade hídrica. Plantas de cana-de-açúcar cresceram em solução nutritiva contendo a mesma concentração de N, porém, variando as proporções de nitrato:amônio em 100:0 e 70:30. Essas proporções foram definidas em experimento prévio, onde avaliamos as respostas da cana-de-açúcar à variação da relação amônio:nitrato e pudemos concluir que as plantas podem ser supridas com até 30% de amônio sem que haja comprometimento da fotossíntese e crescimento. O déficit hídrico foi induzido pela adição de PEG-8000 na solução nutritiva (reduzindo o potencial osmótico de -0,15 para -0,75 MPa). Plantas sob deficiência hídrica e supridas com mais nitrato (100:0) apresentaram maior acúmulo de nitrato e nitrito na raiz, assim como uma maior atividade da NR na raiz, em comparação às plantas supridas com menos nitrato (70:30). Além disso, as plantas supridas apenas com nitrato apresentaram maior produção de NO em raízes, maiores taxas fotossintéticas e condutância estomática, resultando em um maior acúmulo de biomassa. O menor acúmulo de espécies reativas de oxigênio (EROs) encontrado em plantas sob deficiência hídrica supridas apenas com nitrato foi associado ao aumento da atividade da catalase em folhas e da superóxido dismutase e ascorbato peroxidase em raízes. Dessa forma, concluímos que plantas supridas com mais nitrato apresentam maior atividade da NR e produzem mais NO, reduzindo o dano oxidativo causado pelo déficit hídrico e consequentemente favorecendo o metabolismo fotossintético sob baixa disponibilidade de água. Os resultados deste estudo também revelaram que a síntese de NO está associada ao aumento da atividade da NR, sendo uma importante via metabólica em plantas de cana-de-açúcar.

Palavras-chave: Amônio, antioxidantes, enzimas, estresse hídrico, nitrato, nitrogênio, óxido nítrico.

ABSTRACT

Water and nitrogen (N) are two of the most limiting factors in world agricultural production, both strongly associated with the photosynthetic CO_2 assimilation. Modifications in the metabolism of N under water deficit conditions are less studied in C4 plants and the information available is still fragmented. It is known that the radical nitric oxide (NO) is an important signaling molecule in several biological systems, and has a protective role acting mainly against free radicals formed under oxidative stress. Although the mechanisms of NO synthesis and its regulation are complex and poorly understood, nitrate reductase (NR) is a key enzyme for the assimilation of nitrogen in plants and has been proposed as an important enzymatic pathway for NO production through nitrite reduction. Then, the objective of this work was to evaluate the influence of nitrogen nutrition on the physiological responses of sugarcane plants to water deficit, considering the photosynthetic activity, antioxidant metabolism and plant growth. The hypothesis to be tested is that plants receiving more nitrate as source of N will have a higher activity of NR and will produce more NO under water deficit when compared to plants supplied with less nitrate. We expected that NO alleviates the effects of water deficit in sugarcane plants, reducing oxidative damage and improving photosynthesis and plant growth under low water availability. Sugarcane plants were grown in nutrient solution containing the same N concentration, however, varying the proportions of nitrate:ammonium in 100:0 and 70:30. These proportions were defined in a previous experiment, in which we evaluated the responses of sugarcane to the variation of the nitrate:ammonium ratio. We found that plants can be supplied with up to 30% of ammonium without impairment of photosynthesis and growth. Water deficit was induced after adding PEG-8000 to the nutrient solution (reducing the osmotic potential from -0.15 to -0.75 MPa). Plants under water deficit and supplied with more nitrate (100:0) showed a higher root accumulation of nitrate and nitrite, as well as higher root NR activity when compared to plants supplied with less nitrate (70:30). In addition, plants supplied with only nitrate showed higher NO production in roots, higher photosynthetic rates and stomatal conductance, resulting in higher biomass. The lower accumulation of reactive oxygen species (ROS) found in plants under water deficit supplied 100:0 solution was associated with increased activity of catalase in leaves and superoxide dismutase and ascorbate peroxidase in roots. As conclusion, plants supplied with more nitrate presented higher NR activity and produced more NO, reducing the oxidative damage caused by water deficit and consequently favoring photosynthetic metabolism under low water availability. The results of this study also revealed that the NO synthesis is associated with increased NR activity, being an important metabolic pathway in sugarcane plants.

Keywords: Ammonium. antioxidants. enzymes. water stress. nitrate. nitrogen. nitric oxide.

LISTA DE ABREVIATURAS

A_n	Assimilação de CO_2 (μmol m^{-2} s^{-1})
APX	Ascorbato peroxidase
CAT	Catalase
Chl *a*	Clorofila *a*
Chl *a:b*	Razão clorofila *a:b*
Chl *b*	Clorofila *b*
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
eLNC	Concentração estimada de nitrogênio foliar
Ref	Referência
gs	Condutância estomática (mol m^{-2} s^{-1})
GSNOR	S-nitrosoglutationa redutase
k	Eficiência instantânea de carboxilação (μmol m^{-2} s^{-1} Pa^{-1})
MDA	Malondialdeído
NO	Óxido Nítrico
NR	Nitrato redutase
PEPC	Fosfoenolpiruvato carboxilase
RDM	Massa seca da raiz
ROS	Espécies reativas de oxigênio
Rubisco	Ribulose-1,5-bisfosfato carboxilase/oxigenase
RWC	Conteúdo relativo de água
SOD	Superóxido dismutase
SDM	Massa seca da parte aérea
SDM:RDM	Relação da massa seca da parte aérea e raiz
SNO	S-nitrosotiol
WD	Déficit hídrico
WUEi	Eficiência intrínseca do uso da água (μmol mol^{-1})

SUMÁRIO

Introdução Geral

Cana-de-açúcar e déficit hídrico

A cana-de-açúcar é uma cultura de interesse mundial, sendo cultivada em mais de 90 países. O Brasil é o maior produtor mundial de cana-de-açúcar (UNICA, 2017), e a expansão da área de cultivo ocorre principalmente nas regiões Centro-Sul e Centro-Oeste do país, englobando o oeste do estado de São Paulo, sudoeste de Minas Gerais, além dos estados de Mato Grosso do Sul, Mato Grosso e Goiás, conhecidos por apresentarem deficiência hídrica por longos períodos durante o ano (Silva, 2007). A deficiência hídrica é um fator de estresse que promove mudanças em vários aspectos morfológicos, fisiológicos e bioquímicos nas plantas, limitando a produtividade (Boaretto et al., 2014), e assim, para garantir a sustentabilidade da produção, é essencial o entendimento das respostas das plantas às condições estressantes

A cana-de-açúcar é uma cultura relativamente exigente e seu crescimento é altamente sensível ao déficit hídrico (Lakshmanan e Robinson, 2013). As maiores limitações de produtividade da cana-de-açúcar nas áreas cultivadas do Brasil estão relacionadas ao déficit hídrico e se estima que o déficit hídrico pode levar a perdas de produtividade de até 60% (Gentile et al., 2015). As respostas mais comuns ao estresse hídrico na cana-de-açúcar são o enrolamento foliar, o fechamento estomático, a inibição do crescimento dos colmos e folhas, senescência foliar e redução da área foliar (Inman-Bamber et al., 2012). O fechamento estomático pode ser considerado como uma estratégia para evitar a perda de água através da transpiração foliar, no entanto, essa resposta também diminui a disponibilidade de CO_2 para a fotossíntese e a produção de biomassa é reduzida (Machado et al., 2009; Ribeiro et al., 2013). Além disso, foram relatadas reduções no teor de clorofila foliar, inibição da atividade fotoquímica e de enzimas fotossintéticas do metabolismo C4 em cana-de-açúcar sob seca (Machado et al., 2009; Barbosa et al., 2015). Segundo Basnayake et al. (2012), ocorre diminuição na condutância estomática (g_s), na taxa de transpiração (E), na concentração interna de CO_2 (Ci) e na taxa fotossintética (A_n) sob estresse hídrico moderado, principalmente devido à limitação estomática. No entanto, limitação não estomática induzida pelo estresse hídrico também foram relatadas como causa de inibição da fotossíntese na cana-de-açúcar (Ribeiro et al., 2013). Sob estresse hídrico severo, há redução da atividade de enzimas fotossintéticas chaves como a ribulose-1,5-bisfosfato carboxilase/oxigenase (Rubisco) e a fosfoenolpiruvato carboxilase (PEPC) (Lakshmanan e Robinson, 2014). Estas respostas podem estar associadas

com o comprometimento da síntese de ATP e NADPH, reduzindo a regeneração de ribulose-1,5-bisfosfato (RuBP) e fosfoenolpiruvato (PEP) em plantas C4 (Lawlor, 2002), o que é resultado da redução da taxa de transporte de elétrons entre o fotossistema II (PSII) e I (PSI) e de danos nas membranas dos tilacóides (Lawlor e Cornic, 2002). Devido à baixa capacidade de carboxilação e consequentemente, a redução da reciclagem de ATP e NADPH, as plantas enfrentam excesso de energia luminosa e fotoinibição da fotossíntese (Sales et al., 2015).

Devido ao excesso de energia, o estresse hídrico estimula a produção de espécies reativas de oxigênio (EROs), desencadeando estresse oxidativo (Boscolo et al., 2003; Meriga et al., 2004; Sales et al., 2015). EROs são formas reduzidas de oxigênio molecular extremamente reativas, como o ânion superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2), o radical hidroxila ($HO^{\bullet}$) e o oxigênio singleto ($^1O_2$) e são consideradas subprodutos de reações metabólicas nas plantas, como a fotossíntese e a respiração (Ahmad et al., 2010) sendo continuamente produzidas e removidas das células por mecanismos antioxidativos enzimáticos e não-enzimáticos (Mittler, 2002). Dentre as enzimas antioxidantes que participam dos sistemas de defesa das plantas destacam-se a catalase (CAT), a superóxido dismutase (SOD), a ascorbato peroxidase (APX) e a glutationa redutase (GR) (Lázaro et al., 2013), e dentre os metabólitos que podem ser importantes nos sistemas de defesa não-enzimáticos destacam-se o ascorbato e a glutationa (Mittler, 2002; Del Río, 2015). Quando a produção de EROs ultrapassa a capacidade de desintoxicação dos mecanismos antioxidantes, há danos às membranas celulares, DNA e proteínas, o que pode resultar em morte celular (Gill e Tuteja, 2010; Miller et al., 2010).

O controle das funções fisiológicas está diretamente relacionado com o conteúdo hídrico da planta e alterações no teor relativo de água (TRA), afetando diretamente todo o aparato fotossintético. No entanto, as respostas morfológicas e fisiológicas da cana-de-açúcar variam de acordo com o genótipo, o estádio fenológico, a duração (rápida ou gradual) e a intensidade (severa ou leve) do estresse e também do tipo de tecido afetado (Graça et al., 2010; Machado et al., 2010; Inman-Bamber et al., 2012).

Nitrogênio

Assim como a água, o nitrogênio (N) também está fortemente associado à assimilação fotossintética de CO_2, uma vez que é um constituinte vital da clorofila e das enzimas que compõem o aparelho fotossintético (Kumara e Bandara, 2001). Em muitos sistemas de produção, a disponibilidade de N é quase sempre um fator limitante, influenciando o crescimento da planta mais do que qualquer outro nutriente (Bredemeier e Mundstock, 2000).

A aplicação de N no solo aumenta o número e o tamanho das folhas de cana-de-açúcar, o que resulta em um aumento da atividade fotossintética e produtividade (Cha-Um e Kirdmanee, 2008). Hernández et al. (2016) aplicaram diferentes dosagens de fertilizante nitrogenado em plantas de cana-de-açúcar e não notaram diferenças significativas quanto à concentração de N foliar e taxa fotossintética, o que contraria os resultados de Kumara e Bandara (2001). Esses reportaram que a aplicação de fertilizante nitrogenado aumentou significativamente a concentração de N foliar em variedades de cana-de-açúcar, além de um aumento relevante na fotossíntese. Embora conheçamos as respostas das plantas ao N em condições de boa disponibilidade hídrica, as modificações no metabolismo de N que ocorrem durante o déficit hídrico ainda são relativamente pouco estudadas e as informações ainda bastante fragmentadas, sendo a maioria destes estudos realizados em plantas C3.

Os íons NH_4^+ (amônio) e NO_3^- (nitrato) são as formas predominantes de N inorgânico disponível às plantas (Schjoerring et al., 2002). A absorção de NO_3^- e NH_4^+ através da membrana plasmática das células da epiderme e do córtex da raiz ocorre através de transportadores específicos para essas formas de N (Larsson e Ingemarsson, 1989). A absorção de NO_3^- pelas raízes ocorre de forma ativa por simporte, enquanto que o NH_4^+ é absorvido de forma passiva por difusão quando a concentração desse íon na solução do solo é alta (Williams e Miller, 2001). O N, uma vez absorvido, pode ser assimilado na própria raiz ou ser transportado para as folhas, onde, então, ocorre a sua assimilação (Huppe e Turpin, 1994), que compreende os processos de redução do NO_3^- a NH_4^+ e a incorporação do NH_4^+ em aminoácidos. Dessa forma, após ser absorvido, o NO_3^- pode ser reduzido a nitrito (NO_2^-) no citoplasma pela ação da nitrato redutase (NR). Essa enzima é considerada um elemento chave na regulação do metabolismo de N por ser a primeira e principal porta de entrada de N na planta (Purcino et al., 1994). Logo a seguir, o NO_2^- é convertido a amônio NH_4^+ no plastídio, através da enzima redutase do nitrito (RNi) (Masclaux-Daubresseet al., 2010). O NH_4^+ é, então, incorporado em aminoácidos pelas enzimas sintetase da glutamina (GS) e sintase do glutamato (GOGAT), formando glutamina (GLN) e, glutamato (GLU), respectivamente (Crawford, 1995). Alternativamente, o NO_3^- e o NH_4^+ podem ser transportados por carregadores específicos através do tonoplasto e armazenados no vacúolo, para posteriormente serem reduzidos (Crawford, 1995).

A assimilação de N é um processo exigente em termos de energia, e essa exigência varia em função da fonte de N disponível às plantas e dos órgãos da planta onde ele é metabolizado. A assimilação do NO_3^- requer a transferência de 2 elétrons por NO_3^- convertido em NO_2^-, 6 elétrons por NO_2^- convertido em NH_4^+ e 2 elétrons e 1 ATP por molécula de NH_4^+

convertida em GLU (Bloom et al., 1992). Enquanto que a exigência energética da assimilação do NH_4^+ é menor em razão dele não precisar ser reduzido para sua incorporação em aminoácidos (Bredemeier e Mundstock, 2000). No entanto, o NH_4^+ derivado da absorção da raiz é rapidamente assimilado na forma orgânica sendo imediatamente incorporado à aminoácidos nas raízes, isso ocorre pela necessidade de se evitar o seu acúmulo nos tecidos vegetais, uma vez que é considerado tóxico para as plantas (Camargos, 2002). O NO_3^- por sua vez, pode ser acumulado nos vacúolos para posterior utilização ou podem ser translocados através dos tecidos sem efeitos prejudiciais (Lea et al., 1993). Além disso, o NO_3^- acumulado nos vacúolos pode ser considerado importante para os mecanismos de osmoregulação nas plantas (Lasa et al., 2002).

Nas plantas, os íons NO_3^- e NH_4^+ possuem diferentes efeitos no crescimento, na qualidade vegetal, na produção de biomassa e na reprodução (Lane e Bassirirad, 2002). Algumas culturas têm preferência pela absorção de N na forma amoniacal (Malagoli et al. 2000), porém, a maioria dos estudos relatou a redução da produção de biomassa e a ocorrência de sintomas de estresse associados à toxicidade pelo NH_4^+ (Findenegg, 1987; Mengel e Kirkby, 1987). Armas et al. (1992) concluíram que em plantas de cana-de-açúcar podem utilizar tanto NO_3^- quanto NH_4^+ como fonte de N, uma vez que a fotossíntese dessas plantas foi semelhante, independentemente da fonte de N. Já em plantas de pinhão-manso submetidas a estresse salino, houve um aumento nas taxas de assimilação de CO_2 quando houve suprimento de N na forma nítrica (Aragão, et al., 2012). Thomas e Sodek (2005) relataram aumento no peso seco total, altura e área foliar de plantas de soja sob alagamento e supridas com NO_3^-, em comparação com plantas mantidas na ausência de fontes de N exógeno ou na presença de NH_4^+. É fato que o metabolismo de N está envolvido no metabolismo fotossintético, no entanto, cada cultura responde diferentemente em relação à fonte de N disponível e a interação entre o fornecimento de nitrogênio e a ocorrência de estresses ambientais é pouco conhecida.

Óxido nítrico e déficit hídrico

Estudos recentes mostraram que o óxido nítrico (NO) desempenha um papel importante em plantas sob condições estressantes, como a seca (Santisree et al., 2015; Farnese et al., 2016; Silveira et al., 2016; 2017b). O NO é uma molécula reativa e gasosa que interage com diferentes compostos celulares, incluindo outros radicais (Correa-Aragunde et al., 2015), sendo considerado uma importante molécula sinalizadora e com potencial antioxidante (Singh et al., 2009). O NO também participa (i) de uma complexa rede de sinalização celular que induz

o fechamento estomático em resposta ao ácido abscísico (ABA) sob condições de déficit hídrico (Desikan et al., 2002; Bright et al., 2006); (ii) de processos como germinação, crescimento e floração (Farnese et al., 2016); e (iii) da regulação de múltiplas respostas a vários estresses abióticos e bióticos (Neill et al., 2008). Além disso, existem evidências da atuação do NO como molécula de transdução do sinal em vias que levam à indução da resposta de defesa contra ataque de patógenos e a morte celular programada (Delledonne et al., 1998; Hong et al., 2008). Tem-se demonstrado que o NO tem sua síntese aumentada em plantas sob seca (Cai et al., 2015; Silveira et al., 2017a) e se admiti que ele seja um importante mensageiro secundário em plantas (Beligni et al., 2002).

Apesar do grande número de evidências sobre a importância do NO em plantas, o mecanismo responsável pela síntese deste radical é ainda controverso. Em mamíferos, o NO é principalmente sintetizado pela oxidação da L-arginina, catalisada pela óxido nítrico sintase (NOS) (Alderton et al., 2001). Embora haja relatos de uma sintase de óxido nítrico dependente de L-arginina em extratos de diferentes espécies vegetais (Jasid et al., 2006), sua presença em plantas não foi inequivocamente demonstrada (Domingos et al., 2015; Gupta e Igamberdiev, 2015) e um gene homólogo para esta proteína foi encontrado e caracterizado apenas na alga *Ostreococcus tauri* (Moreau et al., 2010; Foresi et al., 2015).

Além da oxidação da L-arginina, hidroxilaminas e poliaminas por enzimas desconhecidas com atividade de NOS, o NO pode também ser produzido pela redução do NO_2^- e mecanismos enzimáticos e não enzimáticos tem sido propostos para esta reação (Figura 1) (Besson-Bard et al., 2008). A enzima NR, além da sua atividade principal de reduzir o NO_3^- a NO_2^-, teria também uma atividade secundária capaz de reduzir o NO_2^- a NO (Rockel et al., 2002; Horchani et al., 2011) durante estresses biótico (Mur et al., 2013) e abióticos, como hipóxia (Igamberdiev e Hill, 2004; Oliveira, et al., 2013), frio (Zhao et al., 2009) e seca (Freschi et al., 2010; Silveira et al., 2017a). A NR pode gerar NO a partir de NO_2^- com NADH como doador de elétrons e o local de catálise ocorre no sítio da enzima composto por um cofator de molibdênio (Harrison, 2002). De acordo com Rockel et al. (2002), a capacidade de produção *in vitro* de NO a partir da NR nas concentrações saturadas de NADH e NO_2^- é de cerca de 1% da sua capacidade de redução de NO_3^-, com a produção *in vivo* de NO dependendo da atividade total da NR, do estado de ativação da enzima e da acumulação intracelular de NO_3^- e NO_2^-. Até o momento, sabemos que NR reduz preferencialmente NO_3^- em NO_2^- sob condições normais de crescimento e que esta enzima só é capaz de gerar quantidades significativas de NO sob certas condições, tais como anaerobiose ou concentrações elevadas de NO_2^- (Gupta et al., 2011; Mur et al., 2013). Oliveira et al. (2013) relataram um aumento na atividade da NR e produção

de NO em segmentos radiculares de soja sob hipóxia suplementados com NO_3^-, porém, o mesmo não foi observado em segmentos radiculares que receberam NH_4^+ como fonte de N. Dentre as vidas redutivas dependentes de NO_2^- para a síntese de NO, há a redução não-enzimática de NO_2^- a NO em pH ácido no apoplasma (Bethke et al., 2004), a produção mitocondrial de NO em que a redução de NO_2^- é impulsionada por elétrons da cadeia de transporte de elétrons (Planchet et al., 2005; Modolo et al., 2005) e pela ação da xantina oxidoredutase (XOR) presente em peroxissomos (Yu et al., 2014) (Figura 1).

O NO pode reagir com o superóxido ($O_2^{\bullet-}$) e produzir o oxidante peroxinitrito ($ONOO^-$) (Yamasaki e Sakihama, 2000), que por sua vez, causa a nitração permanente de resíduos de tirosina (Tyr) em proteínas (Radi, 2002). Embora a nitração de Tyr tenha sido considerada indicativa de estresse, evidências recentes sugerem seu envolvimento na sinalização celular (Mengel et al., 2013). A biodisponibilidade de NO pode ser afetada pela glutationa reduzida (GSH), um antioxidante presente em altas concentrações intracelulares. A GSH pode ser facilmente nitrosada formando S-nitrosotióis (SNOs), como a S-nitrosoglutationa (GSNO) (Barroso et al., 2016), um reservatório natural de NO nas células (Leterrier et al., 2011; Silveira et al., 2016) e componente essencial da via de transdução de sinal dependente de NO (Broniowska et al., 2013). A GSNO pode ser transportada no floema, contribuindo assim para o transporte de NO a longas distâncias, o que desempenha um papel importante na aclimatação sistêmica adquirida (Arasimowicz-Jelonek et al., 2014).

As funções de sinalização de NO e GSNO podem se sobrepor (Silveira et al., 2016) e, a fim de se estabelecer o controle intracelular, a GSNO é catabolizada pela S-nitrosoglutationa redutase (GSNOR) resultando em glutationa oxidada (GSSG) e amônio (Frungillo et al., 2014). Dessa forma, o GSNOR regula os níveis intracelulares de GSNO e é importante na manutenção da homeostase do NO, que é essencial para a sinalização transitória nas células (Malik et al., 2011). Os níveis e atividade da GSNOR são modulados em condições de estresse, sendo importante para a resistência e a aclimatação das plantas (Salgado et al., 2013) e determinados pelo equilíbrio entre EROs e NO (Cheng et al., 2015; Wang et al., 2015; Yang et al., 2015). Porém, o NO derivado da assimilação de NO_3^- pode inibir a GSNOR por S-nitrosilação, evitando a remoção de GSNO. Dessa forma, GSNO controla sua própria geração e eliminação através da modulação da assimilação de NO_3^- e da atividade de GSNOR (Chamizo-Ampudia et al., 2017; Frungillo et al., 2014).

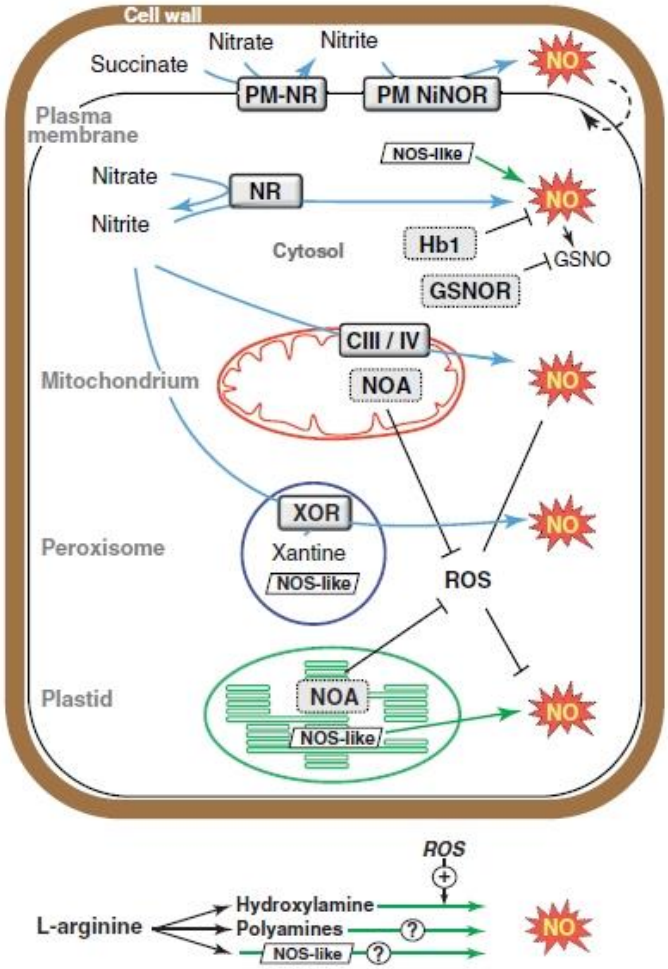

Figura 1. Visão geral das vias biossintéticas do NO e sua localização subcelular nas plantas. A figura mostra uma representação esquemática de uma célula vegetal com os compartimentos subcelulares nos quais ocorrem as vias de produção de NO. NOS-like: produção de NO catalisada pela enzima com atividade semelhante à NOS animal; NOA: proteína associada ao NO; NR: nitrato redutase; PM-NR e NiNOR: NR e NiNOR ligado à membrana plasmática; ?: enzimas não identificadas; XOR: xantina oxidoredutase; GSNO: s-nitrosoglutationa; GSNOR: s-nitrosoglutationa redutase; ROS: espécies reativas de oxigênio. As setas em azul são vias redutoras e as setas em verde indicam vias de reação oxidativa (Gupta et al., 2011).

A S-nitrosilação é um processo pelo qual ocorre interação de NO a resíduos Cys de proteínas, levando a formação de RSNO (Besson-Bard et al., 2008; Astier et al., 2012), e aparentemente é o principal mecanismo para a transdução da bioatividade de NO (Lamotte et al., 2015). A S-nitrosilação pode alterar a atividade, estabilidade e a conformação de proteínas alvo, além de interações com outras moléculas ou sua localização subcelular, regulando uma vasta gama de funções celulares e eventos de sinalização (Sevilla et al., 2015) e sendo um

processo importante na resposta das plantas a estresses abióticos. A exposição ao estresse salino, por exemplo, resulta na S-nitrosilação de enzimas envolvidas em diferentes processos fisiológicos, como respiração, fotorrespiração e vias antioxidantes (Camejo et al., 2013). Em plantas expostas a baixa temperatura, as enzimas envolvidas no metabolismo de carbono foram o principal grupo de proteínas S-nitrosiladas (Puyaubert et al., 2014). Adicionalmente, a GSNO é capaz de transferir diretamente a sua fração NO para grupos tiol de proteínas, um processo referido como S-transnitrosilação (Salgado et al., 2013) e assim, tanto o NO como a GSNO são capazes de controlar a atividade de proteínas (Yu et al., 2014).

A produção de NO induzida pela seca foi encontrada em várias espécies de plantas, sugerindo a exigência de NO durante a sinalização de estresse por seca (Santisree et al., 2015). As plantas tolerantes à seca costumam ter um controle rigoroso dos movimentos estomáticos e um equilíbrio fino do metabolismo celular, e tanto as EROs como o NO são importantes nesses processos (Osakabe et al., 2014). Uma das primeiras e mais importantes respostas fisiológicas induzidas pela seca é a redução da abertura estomática (Neill et al., 2008). Em condições de déficit hídrico, a dinâmica do movimento estomático está diretamente relacionada à concentração de ABA, EROs e NO. Neste processo, o ABA atua como um regulador, induzindo a síntese de NO, que por sua vez, junto aos EROs agem sinergicamente para mediar o fechamento estomático através da formação de 8-nitro-cGMP (Joudoi et al., 2013), que é um novo elemento na sinalização de plantas em condições estressantes.

Evidências farmacológicas e genéticas indicam que a sinalização mediada por NO aumenta a tolerância ao estresse hídrico (Tian e Lei, 2006; Cai et al., 2015; Foresi et al., 2015), como observado em *Vicia faba* (Garcia-Mata e Lamattina, 2001). Após pulverizar GSNO (doador de NO) em folhas de cana-de-açúcar, Silveira et al. (2016) também notaram uma melhora na tolerância da cana-de-açúcar ao déficit hídrico e aumento das taxas fotossintéticas. A melhora na assimilação CO_2 foi associada, em parte, com o maior teor relativo de água e maior condutância estomática sob déficit hídrico. Além da limitação difusiva da fotossíntese imposta pelo fechamento estomático, as plantas podem sofrer limitações bioquímicas sob seca severa. Silveira et al. (2017b) notaram que a GSNO exógeno melhora a captação de CO_2 sob déficit hídrico pelo aumento na atividade de Rubisco em plantas de cana-de-açúcar. Adicionalmente, o NO induz um aumento lento e contínuo do coeficiente de extinção não fotoquímico da fluorescência, um mecanismo fotoprotetor bem conhecido (Ordog et al., 2013). Alternativamente, o desempenho melhorado de plantas sob seca e supridas com doador de NO pode ser devido à redução dos danos oxidativos (Silveira et al., 2017b).

A biossíntese e a degradação de EROs e NO influenciam-se mutuamente (Groß et al., 2013). As EROs são indutoras da síntese de NO em várias espécies de plantas expostas ao estresse abiótico, e o NO por sua vez, limita a acumulação de EROs pela redução da atividade de NADPH oxidases, proteínas integrais de membrana responsáveis pela formação de $O_2^{\bullet -}$ e por promover a acumulação de EROs no apoplasto (Das e Roychoudhury, 2014), e também por promover mudanças na atividade do sistema antioxidante (Groß et al., 2013). De fato, a ativação de mecanismos antioxidantes para manter a homeostase de EROs envolve frequentemente a participação de NO (Shi et al., 2014; Hatamzadeh et al., 2015; Silveira et al., 2015). O efeito protetor da aplicação exógena de moléculas doadoras de NO tem sido atribuído à eliminação de $O_2^{\bullet -}$ e ao aumento nas atividades de enzimas antioxidantes em cana-de-açúcar (Silveira et al., 2017b). Também foi demonstrado que a aplicação de NO aumentou a atividade de superóxido dismutase (SOD) em até 110% em plantas de sorgo expostas ao arsênio (Saxena e Shekhawat, 2013), além do aumento das atividades da catalase (CAT) e ascorbato peroxidase (APX) e da ativação do ciclo ascorbato-glutationa (Hasanuzzaman e Fujita, 2013; Shi et al., 2014; Cheng et al., 2015).

Objetivo e Hipóteses

Esse estudo abordará questões que envolvem a disponibilidade de água e o crescimento de uma cultura bioenergética de interesse estratégico para o país. Pretende-se revelar se a fonte de nitrogênio tem influência nas respostas fisiológicas de cana-de-açúcar frente ao déficit hídrico. Vislumbra-se que a simples escolha da fonte de nitrogênio ou variações entre as fontes, no momento da adubação para o plantio de cana-de-açúcar poderia tornar a cultura menos suscetível ao déficit hídrico durante o desenvolvimento inicial, período crucial e de maior suscetibilidade à seca (Machado et al., 2009).

A hipótese desse estudo é que plantas que recebem mais nitrato como fonte de nitrogênio terão uma maior atividade da nitrato redutase e em situação de déficit hídrico produzirão mais óxido nítrico, quando comparadas as que recebem menos nitrato. Espera-se que o NO desempenhe um papel atenuador do déficit hídrico em plantas de cana-de-açúcar, reduzindo o dano oxidativo causado pelas espécies reativas de oxigênio e melhorando a fotossíntese e o crescimento das plantas sob baixa disponibilidade hídrica.

O estudo é composto por três experimentos, cujos resultados estão organizados em dois capítulos. No primeiro capítulo, realizamos um estudo preliminar com o intuito de avaliar as respostas da cana-de-açúcar à variação da relação amônio:nitrato. Para isso, plantas de cana-de-açúcar foram cultivadas em solução nutritiva com relações crescentes de amônio:nitrato (de 20 a 80%). Com os resultados obtidos, foi possível chegar a um valor máximo de amônio que pode ser fornecido à essas plantas sem que haja comprometimento da fotossíntese e do crescimento. Esses resultados foram fundamentais para a realização do segundo experimento, descrito no segundo capítulo, onde testamos a hipótese de que plantas de cana-de-açúcar que recebem mais nitrato como fonte de nitrogênio terão uma maior atividade da nitrato redutase e em situação de déficit hídrico produzirão mais óxido nítrico, quando comparadas as que recebem menos nitrato. Um terceiro experimento, descrito no segundo capítulo ainda, foi realizado com o objetivo de confirmar os efeitos mediados pelo NO observados em condições de déficit hídrico no segundo experimento.

Capítulo 1

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Photosynthesis and biomass accumulation in young sugarcane plants grown under increasing ammonium supply in nutrient solution

Running title: Sugarcane sensitivity to NH_4^+

Photosynthesis and biomass accumulation in young sugarcane plants grown under increasing ammonium supply in nutrient solution

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Abstract

The aim of this study was to evaluate the sugarcane responses to varying ammonium:nitrate (NH_4^+:NO_3^-) ratio and to reveal how much NH_4^+ plants can tolerate before showing impairment of photosynthesis and growth. Sugarcane plants were grown in nutrient solution with the following NH_4^+:NO_3^- ratios (%): 20:80; 30:70; 40:60; 60:40; 70:30; and 80:20. The lowest photosynthetic rates, stomatal conductance, instantaneous carboxylation efficiency and leaf chlorophyll *a* content were found in plants supplied with higher than 60% NH_4^+. The leaf content of chlorophyll *b* proved to be more sensitive than chlorophyll *a* and decreases were found from 40% NH_4^+. We did not observe significant differences in leaf NO_3^- concentration under varying NH_4^+:NO_3^- ratio. However, plants that received 80% NH_4^+ showed the highest leaf NH_4^+ concentration and lowest leaf [NO_3^-]:[NH_4^+] ratio. The estimated leaf nitrogen content was higher in plants supplied with 20% and 30% NH_4^+. Taken together, our data revealed that sugarcane plants are sensitive to NH_4^+, with photosynthesis and plant growth being impaired when NH_4^+ supply was higher than 30% in nutrient solution. Root biomass was significantly reduced under high NH_4^+ supply, which explains decreases in stomatal conductance. Besides stomatal limitation, photosynthesis was also limited by low carboxylation efficiency under high NH_4^+ supply. Apparently, leaf NH_4^+ concentrations higher than 1.0 µmol g^{-1} were enough to impair photosynthesis. The balance between [NO_3^-] and [NH_4^+] in leaves was more correlated to photosynthesis than either [NO_3^-] or [NH_4^+] alone.

Keywords: Nitrogen, Root growth, *Saccharum* spp, Nitrate

Introduction

Nitrogen (N) is a key element for plant growth and development, being indispensable for protein synthesis and plant metabolism (Piwpuan et al., 2013). In general, N is taken up by roots either as nitrate (NO_3^-) or ammonium (NH_4^+), following assimilatory pathways with varying energetic costs (Luo et al., 2013). The energetic demand for reducing NO_3^- to NH_4^+ through the N assimilatory pathway is high and plants can obtain such energy from either photoassimilate consumption in roots and non-photosynthetizing tissues or from the primary photochemical reactions occurring in leaves (Araújo et al., 2012). When comparing forms, NH_4^+ assimilation needs less energy than NO_3^- assimilation as the steps to convert NO_3^- into NO_2^- and NO_2^- into NH_4^+ are bypassed (Bittsánszky et al., 2015).

While NO_3^- is mobile and can be stored inside cell vacuoles without causing any toxicity to plants (Lasa et al., 2002), NH_4^+ is toxic when its concentration reaches a critical level in cells (Findenegg, 1987). Most of the NH_4^+ is assimilated in roots after passive uptake through low-affinity transporters and high NH_4^+ influx is unavoidable when high NH_4^+ prevails in root media (Wang et al., 1994). Besides toxicity induced by its uptake (Britto and Kronzucker, 2002), NH_4^+ can also accumulate within cells when environmental stresses enhance protein degradation and cause NH_4^+ overproduction (Skopelitis et al., 2006; Hirel et al., 2007).

As a consequence of toxic NH_4^+ levels, hormonal homeostasis is disrupted and photophosphorylation reduced (Coskun et al., 2013), leading to cytosol acidification due to high NH_4^+ and H^+ contents and consequently hyperpolarization of membranes (Britto et al., 2001). Such conditions cause an oxidative burst within cells and reactive oxygen species damage the overall cell structure (Li et al., 2014). Besides reducing the efficiency of the photochemical apparatus due to membrane damage, NH_4^+ accumulation also decreases the concentration of photosynthetic pigments (Wang et al., 2010) and leaf gas exchange (Su et al., 2012; Borgognone

et al., 2013). As a result, plants exhibit leaf chlorosis and stunted roots due to NH_4^+ toxicity (Li et al., 2014), with plant death occurring under severe stress (Jampeetong et al., 2012).

Although NO_3^- and NH_4^+ can affect differentially plant metabolism and growth (Lane and Bassirirad, 2002), De Armas et al. (1992) found that sugarcane plants were insensitive to N sources and no differences in photosynthesis were found when comparing plants supplied exclusively with NO_3^- or NH_4^+. In fact, there is no consensus concerning nitrogen supply for plants and Mariano et al. (2015) do not recommend NH_4^+ as sole nitrogen source for sugarcane as this source did not increase sugarcane yield compared to the control (no N added). Robinson et al. (2011) evaluated the recovery of ^{15}N by sugarcane plants and concluded that when NO_3^- and NH_4^+ are in similar and high concentration in nutrient solution, sugarcane strongly prefer NH_4^+ in relation to NO_3^-. Boschiero et al. (2018) also found that NH_4^+ was preferentially absorbed by sugarcane but this pattern changed after some time and the highest ^{15}N recovery was detected in plants supplied with NO_3^-. Although the uptake of NH_4^+ is faster as compared to NO_3^-, it is not necessarily translated into higher ^{15}N recovery in long term. Sugarcane plants supplied with only NO_3^- showed high N utilization efficiency and high biomass production and leaf gas exchange than those supplied with only NH_4^+ (Boschiero et al., 2019). While Physic nut plants presented higher photosynthetic rates when supplied with NO_3^- (Aragão et al., 2012) and spinach growth was impaired under NH_4^+ supply (Domínguez-Valdivia et al., 2008), *Cana indica* exhibits higher photosynthesis when receiving NH_4^+ instead of NO_3^+ as nitrogen source (Konnerup and Brix, 2010). When considering the interaction with water availability, rice plants had improved drought tolerance when supplied with NH_4^+ instead of NO_3^+ (Cao et al., 2018).

Current knowledge about plant nutrition led us to assume that plants are sensitive to NH_4^+ and that the critical NH_4^+ level causing damage varies among species. Here, our aim was

to evaluate sugarcane responses to varying NH_4^+:NO_3^- ratios and reveal how much NH_4^+ plants can tolerate before showing impairment of photosynthesis and growth.

Materials and Methods

Plant material, growth conditions and treatments

Sugarcane (*Saccharum* spp.) plants cv. IACSP95-5000 were propagated by using culm segments (with one bud) in 0.5 L plastic pots containing commercial substrate composed of *Sphagnum*, rice straw and perlite (7:2:1, Carolina Soil of Brazil, Vera Cruz RS, Brazil). After 30 days, plants were transplanted to 4 L plastic boxes containing nutrient solution proposed by De Armas et al. (1992) modified to supply 2 mmol N L^{-1} while varying NH_4^+:NO_3^- ratio using NH_4Cl and KNO_3 as N sources, respectively. Changes in NH_4^+:NO_3^- ratios were balanced and concentrations of Cl^- varied from 2.7 to 3.3 µmol L^{-1} and K^+ from 3.2 to 3.5 mmol L^{-1}. The nutritive solution also contained: 1.9 mmol Ca L^{-1}; 0.5 mmol Mg L^{-1}; 1.2 mmol P L^{-1}; 1.2 mmol S L^{-1}; 24.0 µmol B L^{-1}; 16 µmol Fe L^{-1}; 9 µmol Mn L^{-1}; 3.5 µmol Zn L^{-1}; 1 µmol Cu L^{-1}; and e 0.1 µmol Mo L^{-1}. Under the same level of supplied nitrogen (2 mmol N L^{-1}), plants were grown with varying NH_4^+:NO_3^- ratios, as follows (in %): 20:80; 30:70; 40:60; 60:40; 70:30; and 80:20. We transferred four plants per box, totaling four replicates per treatment. Before transfer of plants to the nutrient solution, roots were washed with tap water to remove any adhering substrate. The modified De Armas' nutrient solution was diluted at ¼ strength at the beginning. After four days the nutrient solution was renewed and its strength increased to ½. After another four days full strength solution was supplied to the plants, where they remained for another four days (total of 12 days of acclimation to the solution) and the experiment was began and lasted for 21 days.

The nutrient solution was renewed every three days throughout the experimental period, with electrical conductivity maintained between 1.2 and 1.5 mS cm^{-1} and pH 5.9±0.1. The pH was adjusted daily with 0.5 M ascorbic acid or 0.5 M NaOH. Both variables were monitored on a daily basis using a portable conductivity meter (mCA 150P, MS Tecnopon Instrumentação, Piracicaba SP, Brazil) and a portable pH meter (mPA 210P, MS Tecnopon Instrumentação, Piracicaba SP, Brazil). The volume of the nutrient solution was also checked on daily basis and completed with water when necessary. The solution in each box was continuously aerated by an air compressor (Master Super II, Master, São Paulo SP, Brazil). Plants were grown under greenhouse conditions, where air temperature varied between 33.3 and 15.5 ºC, the minimum and maximum air relative humidity was 55% and 96%, respectively, and the maximum photosynthetic active radiation (PAR) reached 1500 µmol m^{-2} s^{-1}.

Leaf gas exchange

Leaf gas exchange was measured with an infrared gas analyzer model LCi-SD (ADC BioScientific Ltd., Hoddesdon, UK), and data recorded after temporal stability. Leaf CO_2 assimilation (A_n), stomatal conductance (g_s), transpiration (E) and intercellular CO_2 concentration (C_i) were measured during the experimental period on the first fully expanded leaf with visible dewlap (leaf +1). Measurements were taken between 10h30 and 12h30, under PAR of 1450±167 µmol m^{-2} s^{-1}, an air temperature about 32±1 ºC, air CO_2 concentration of 380 µbar and air relative humidity of 65.9 ± 6.8%. We did not use supplementary light and light intensity varied naturally during the measurements. Intrinsic water use efficiency (WUEi) and instantaneous carboxylation efficiency (k) were estimated as A_n/g_s and A_n/C_i, respectively (Machado et al., 2009).

For the biochemical analyses, the leaves +1 were collected on the last day of evaluation (21st day) and immediately immersed in liquid nitrogen and then stored at -80 °C.

Leaf NO_3^- and NH_4^+ concentrations

Fresh leaf samples (0.5 g) were ground in liquid nitrogen and 2.5 mL of extraction medium containing methanol:chloroform:water (12:5:3 v/v) added to the powder. After centrifugation at 2000 *g* for 5 min, the supernatants were collected and 1 mL of chloroform and 1.5 mL of water were added to 2 mL of the supernatant. The extract was centrifuged again for 3 min at 2000 *g* for phase separation. The upper aqueous phase was collected and maintained in a water bath at 37 °C for two hours to remove traces of chloroform and then the extracts were stored at -20 ºC (Bieleski and Turner, 1966).

Nitrate: 50 µL of the extract were pipetted into test tubes and 200 µL of reaction medium (5% w/v salicylic acid + concentrated sulfuric acid) were added. After 20 min, 4.75 mL of 2N NaOH were added and the solution stirred. After cooling at room temperature, the absorbance was read in a spectrophotometer (Ultrospec 1000, Pharmacia Biotech, Cambridge, England) at 410 nm and the nitrate content was calculated from a standard curve using KNO_3 (100-1000 nmol) (Cataldo et al., 1975).

Ammonium: 50 µL of the extract were pipetted into microtubes. Then, 250 µL of solution A (1% phenol + 0.005% sodium nitroprusside) were added followed by 250 µL of solution B (0.5% sodium hydroxide containing 2.52% sodium hypochlorite). The tubes were kept for 35 min in a water bath at 37 °C and the absorbance read at 625 nm after cooling to room temperature (McCullough, 1967). A standard curve of $(NH_4)_2SO_4$ (0-100 mmol) was used to estimate the ammonium content.

Chlorophyll and estimation of leaf N concentration

After 21 days of treatment, the relative content of chlorophylls (Chl) *a* and *b* was measured with a chlorophyll meter (CFL1030, Falker, Porto Alegre RS, Brazil) in the same leaves used for gas exchange measurements. Estimation of leaf N concentration (eLNC) was based on Chl *b* readings, following Cerqueira et al. (2019): eLNC=[1.488+2.133*(Chl *b*)–0.049*(Chl *b*)²].

Plant growth

After 21 days growing under varying NH_4^+:NO_3^- ratios, the dry mass (DM) of leaves, culm and roots was evaluated after drying samples in a forced air oven at 60 °C. The shoot:root DM ratio was calculated.

Experimental design and statistical analysis

The experimental design was in randomized blocks containing six treatments, four blocks and one plant per replicate. Data were subjected to the analysis of variance (ANOVA). When statistical significance was detected, the mean values (n=4) of treatments were compared by the Tukey test (p<0.05), using the software Assistat version 7.7 (UFCG, Campina Grande PB, Brazil). Correlations among variables were evaluated using the software OriginPro 8 SR0 version 8.0724 (OriginLab Corporation, Northampton MA, USA).

Results

Leaf gas exchange was significantly affected by changing NH_4^+:NO_3^- ratios in the nutrient solution (Fig. 1). After 9 days of treatment plants showed the highest photosynthesis (A_n) when the NH_4^+ proportion ranged from 20% to 40% of total N (Fig. 1a). While the lowest photosynthetic rates and stomatal conductance (g_s) were found in plants at 60%, 70% and 80% of NH_4^+, the highest values of g_s occurred in plants receiving 20% NH_4^+ (Fig. 1a,c). In general, g_s was more sensitive to NH_4^+ than A_n. Increases in intrinsic water use efficiency (WUEi) were found with increasing the NH_4^+:NO_3^- ratio but there was no clear and significant trend among treatments (Fig. 1b). Regarding the instantaneous carboxylation efficiency (k), plants receiving less than 40% NH_4^+ presented the highest k values (Fig. 1d). After 21 days of treatment, we found that A_n, g_s and the k were similar ($p>0.05$) between 20% and 40% of NH_4^+, showing a decrease after this threshold (Fig. 2).

Leaf NO_3^- concentration did not vary significantly when increasing NH_4^+ proportion (Fig. 3a). On the other hand, leaf NH_4^+ concentration showed a sudden increase when the proportion of NH_4^+ was higher than 60% (Fig. 3b). Accordingly, $[NO_3^-]$:$[NH_4^+]$ ratio decreased with increasing NH_4^+ proportion, with the lowest leaf $[NO_3^-]$:$[NH_4^+]$ ratios (~1.4) found under 80% NH_4^+ (Fig. 3c). A reduction in the estimated leaf nitrogen concentration (eLNC) occurred when NH_4^+ proportion exceeded 30% (Fig. 3d).

The leaf concentration of Chl *b* was more sensitive than Chl *a* to increasing NH_4^+ supply, with significant decreases in both Chl *a* and *b* being found when NH_4^+ proportions were higher than 40% (Fig. 4a, b). As a consequence, the Chl *a:b* ratio was increased from 30% of NH_4^+ (Fig. 4c).

While g_s and k were positively correlated ($p<0.05$) to leaf $[NO_3^-]$, only A_n was negatively correlated ($p<0.05$) to leaf $[NH_4^+]$ (Table 1). Interestingly, all leaf gas exchange variables were

positively and significantly correlated to leaf $[NO_3^-]$:$[NH_4^+]$ ratio, with *r*-values ≥ 0.85 and *p*-values ≤ 0.02 (Table 1). eLNC was better correlated to g_s and *k* than any other N-related variable (Table 1). A poor correlation between leaf A_n and eLNC was found (Table 1).

Plant dry mass (DM) production was reduced by NH_4^+ supply and such effects were more evident for the root system (Fig. 5a). While the accumulation of leaf and culm biomass was reduced when the NH_4^+ proportion varied between 60% and 80%, root biomass was decreased under 40% NH_4^+ (Fig. 5b). As a consequence of the differential sensitivity of plant organs to NH_4^+, the shoot:root ratio increased with increasing NH_4^+ supply (Fig. 5c).

Discussion

Our findings revealed that sugarcane growth was impaired when young plants were supplied with high ammonium proportion in nutrient solution, with decreases in root biomass and consequent increases in shoot:root ratio when grown in nutrient solution with more than 30% of NH_4^+ (Fig. 5b, c). Shoots were less sensitive to NH_4^+ than roots, which is in agreement with a previous study with maize plants (Bennett et al., 1964). More recently, Boschiero et al. (2019) observed that sugarcane plants supplied with NH_4^+ showed shoot and root dry mass 20% and 38% lower than those supplied with NO_3^-, respectively. Hajari et al. (2015) also found lower root dry mass in sugarcane plants supplied NH_4^+ compared to those ones supplied with NO_3^-, which also resulted in increased shoot:root ratio.

Interestingly, Australian sugarcane cultivars show a strong "preference" (i.e., higher uptake efficiency) for NH_4^+ uptake over NO_3^- during the first two days after fertilization (Robinson et al., 2011). Three possible factors might explain the higher uptake rate of NH_4^+ in relation to NO_3^-: (a) rapid influx of NH_4^+ into the roots (Britto et al., 2001); (b) the energy cost for uptake and assimilation of NH_4^+ is lower than that of NO_3^- (Britto and Kronzucker, 2005);

and (c) NO_3^- uptake is delayed by exposure to high NH_4^+ concentration (Aslam et al., 2001). Thus, the term "preference for NH_4^+" is based on initially higher uptake rate of NH_4^+ as compared to NO_3^- (Boschiero et al., 2018), which does not mean that NH_4^+ is beneficial to sugarcane. A proper assessment of how N sources can affect plants must take into account biomass production and N use efficiency (NUE) (Good et al., 2004). Hajari et al. (2015) detected higher NUE for NO_3^- than NH_4^+ in three sugarcane cultivars *in vitro* conditions, while Boschiero (2017) reported higher dry biomass and NUE in sugarcane plants supplied exclusively with NO_3^- than those only receiving NH_4^+ or different ratios of both N sources. Here, the highest root dry mass and eLNC were found in plants receiving 70% to 80% of NO_3^- (Fig. 5b; Fig. 3d).

As NH_4^+ nutrition tends to acidify the rhizosphere (Britto et al., 2001), the pH of our nutrient solution was monitored daily and corrected when necessary to exclude the influence of solution acidity. Then, the reduction plant growth under high NH_4^+ could be primary associated to inability in controlling the influx of NH_4^+ into the cells when the concentration of such ion is excessive in root medium. Under such a condition, an efflux mechanism – also known as futile cycling – is activated involving high energy expenditure to pump NH_4^+ out of the cell, which increases the maintenance respiration (Britto and Kronzucker 2002). In addition, NH_4^+ is preferentially assimilated in the roots (Hachiya et al., 2010), a mechanism that explains high root sensitivity to this ion. The low photosynthetic rates would be another factor causing reduction of plant growth when in the presence of over 30% of NH_4^+ in nutrient solution (Figs. 2a, 5b).

Herein, the highest photosynthetic rates were found in plants that received up to 40% of NH_4^+, with a sharp decline in photosynthetic rates after this threshold (Fig. 2a). Although CO_2 assimilation is related to high stomatal conductance and instantaneous carboxylation efficiency, stomatal conductance was more sensitive than photosynthesis to increasing NH_4^+ (Fig. 2a-c).

Even with such higher sensitivity of the stomatal apparatus to NH_4^+, stomatal aperture does not seem to be limiting since changes in stomatal conductance did not reduce photosynthesis under low NH_4^+ supply (Fig. 2a, b). The reduction of stomatal conductance could be partially associated with low leaf K concentration. Potassium plays a crucial role in regulating the turgor of guard cells (Blatt et al., 2014) and its likely deficiency under high NH_4^+ supply would be explained by competition between K^+ and NH_4^+. The K-specific channels may also mediate NH_4^+ uptake into the root cells (Balkos et al., 2010; Coskun et al., 2013), causing low K uptake. As consequences of K deficiency, stomatal closure, low photosynthetic rates, reduced synthesis of organic acids have been found in several plant species (Von Wirén et al., 2001; Jin et al., 2011).

As a consequence of the stomatal conductance reduction, we found increases in intrinsic water use efficiency (WUEi) at the leaf level (Fig. 1b), a response that would benefit plants growing in rainfed areas. Since eLNC was also reduced under high NH_4^+ supply (Fig. 3d; Table 1) and aquaporin expression is decreased under low N availability (Ishikawa-Sakurai et al., 2014; Ding et al., 2018), the stomatal closure found herein (Fig. 2b) could be a consequence of low plant hydraulic conductance. Aquaporins not only respond to the availability of N, but also to their forms, including NH_4^+ and NO_3^- (Gao et al., 2018). Guo et al. (2007) reported that aquaporin expression was lower when common bean plants were supplied with NH_4^+ instead of NO_3^-, with roots exposed to NO_3^- showing higher hydraulic conductance. Herein, poor root development under high NH_4^+ supply may be the cause of low stomatal conductance (Figs. 2b, 5a), as a result of low water uptake that did not match the atmospheric demand.

Nitrogen fertilization affects the hydraulic architecture of plants, with changes in dry mass partitioning to the root system, which is a long-term response to varying N sources (Siemens and Zwiazek, 2013). As such modifications have implications for water transport – and then nitrogen transport, one would expect changes in plant growth and drought tolerance

due to the N source. Accordingly, Faustino et al. (2015) reported changes in xylem morphology and a strong modulation of root hydraulic conductivity in *Pinus* plants supplied with NO_3^-, which alleviate the impact of drought on plant growth, a response not observed in plants supplied with NH_4^+.

The instantaneous carboxylation efficiency (*k*) was also highly correlated with eLNC (Table 1) and both variables were reduced under high NH_4^+ supply (Figs. 2c and 3d). Such a correlation between *k* and eLNC is expected since a considerable fraction of N is directed towards photosynthetic metabolism and synthesis of chlorophylls and the key enzymes ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase (Sinclair and Horie, 1989). While total leaf N content correlates well with the activity of the above enzymes (Sage and Pearcy, 1987), photosynthesis increases linearly with increasing leaf N content in sugarcane (Meinzer and Zhu, 1998). Thus, our data suggest that the reduction of eLNC in plants supplied with more than 40% NH_4^+ could affect the synthesis of key photosynthetic enzymes and thereby explain decreases in the instantaneous carboxylation efficiency (Fig. 2c).

With regard to the chlorophyll *a* and *b* contents, we observed a significant reduction when supplying plants with more than 40% NH_4^+ (Fig. 4a,b), which may also explain lower photosynthetic rates. When the root nitrogen assimilation capacity is exceeded under high NH_4^+ supply, this ion accumulates in roots and is further translocated to leaves (Britto and Kronzucker, 2002). Accumulation of NH_4^+ in leaves is related to photosynthetic impairment (Guo et al., 2006). The highest leaf NH_4^+ concentrations were found in plants supplied with 70% and 80% NH_4^+ (Fig. 3b). As the negative effects of NH_4^+ were verified when its proportion in nutrient solution was higher than 40%, our data indicate that NH_4^+ was fully metabolized since its concentration in leaves remained unchanged when supplying plants with 40% and 60% NH_4^+ (Fig. 3b). The assimilation of NH_4^+ is less costly than NO_3^- (Bloom et al., 1992), the

above finding could be an advantage in energetic terms as long as the leaf NH_4^+ concentration does not reach toxic levels (> 1.0 μmol g^{-1}) or the leaf $[NO_3^-]$:$[NH_4^+]$ ratio is maintained lower than 2 (Fig. 3b,c). None significant change was found for leaf NO_3^- concentration while varying the proportion of N sources (Fig. 3a). Although plants metabolize both NO_3^- and NH_4^+, the former can be stored at high levels and translocated among tissues (Guan et al., 2016).

According to Trivelin et al. (2013), nitrogen fertilization is one of the most important factors in sugarcane cultivation, with urea being the nitrogen fertilizer most commonly used in crop systems. However, with urea can occurs losses of 20 to 40% of the N applied through volatilization of ammonia (Cantarella et al., 2008). Thus, alternative sources of N for promoting plant growth and development while reducing losses through volatilization would be beneficial for both crops and environment (Zhao et al., 2010). In the sugarcane fields, the response to nitrogen fertilization is still an issue that needs to be further explored. As an example, we have recently observed that leaf N supply reduced the sensitivity of sugarcane photosynthesis to cold (Cerqueira et al., 2019), which could favor sucrose accumulation in culms during the cold and dry winter in subtropical regions. In addition, recent studies have shown an increase in drought tolerance of sugarcane induced by nitric oxide (Silveira et al., 2016; 2017a, b; 2019). Nitric oxide is produced after the reduction of nitrite, using NADPH (Rockel et al., 2002; Yamasaki and Sakihama, 2000). On the other hand, nitrite is a product of nitrate reduction through nitrate reductase (Katajisto et al., 2015). While NH_4^+ supply has a toxicity potential for sugarcane plants (Mariano et al., 2015) and there are large N losses in crop fields fertilized with ammonium (Zhao et al., 2010; Trivelin et al., 2013), NO_3^- supply could supply plant N demand while improving drought tolerance through NO synthesis, a hypothesis that must be tested.

Conclusion

Young sugarcane plants were sensitivity to ammonium in nutrient solution, with photosynthesis and plant growth being impaired when the proportion of NH_4^+ as nitrogen source in a mixture with NO_3^- was higher than 30%. The root system was the most sensitive plant organ to increasing NH_4^+ supply, with reductions in root biomass causing decreases in stomatal conductance. Besides low stomatal conductance, photosynthesis was also limited by low carboxylation efficiency under high NH_4^+ supply. Leaf NH_4^+ concentrations higher than 1.0 μmol g^{-1} were enough to cause impairment of photosynthesis whereas leaf $[NO_3^-]$:$[NH_4^+]$ ratio explained changes in photosynthesis better than NO_3^- or NH_4^+ concentrations alone.

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Table 1. Pearson correlation coefficient (r) and correlation significance (p) between leaf gas exchange variables [leaf CO_2 assimilation (A_n), stomatal conductance (g_s) and instantaneous carboxylation efficiency (k)] and leaf concentrations of NO_3^-, NH_4^+ and estimated of leaf N concentration (eLNC) and $[NO_3^-]$:$[NH_4^+]$ ratio in sugarcane plants growing under increasing NH_4^+ supply.

Variables	$[NO_3^-]$	$[NH_4^+]$	$[NO_3^-]$:$[NH_4^+]$	eLNC
A_n	$r = 0.72$ $p = 0.067$	**$r = -0.82$** **$p = 0.029$**	**$r = 0.85$** **$p = 0.020$**	$r = 0.72$ $p = 0.063$
g_s	**$r = 0.86$** **$p = 0.018$**	$r = -0.72$ $p = 0.065$	**$r = 0.89$** **$p = 0.010$**	**$r = 0.90$** **$p = 0.009$**
k	**$r = 0.80$** **$p = 0.035$**	$r = -0.67$ $p = 0.088$	**$r = 0.86$** **$p = 0.018$**	**$r = 0.93$** **$p = 0.004$**

Bold numbers indicate significant correlation ($p<0.05$)

Figure captions

Figure 1. Temporal dynamics of leaf CO_2 assimilation (A_n, in a), intrinsic water use efficiency (WUEi, in b), stomatal conductance (g_s, in c), and instantaneous carboxylation efficiency (k, in d) of sugarcane plants under increasing NH_4^+:NO_3^- ratios in nutrient solution. Symbols represent the mean value ± se (n=4).

Figure 2. Leaf CO_2 assimilation (A_n, in a), stomatal conductance (g_s, in b), and instantaneous carboxylation efficiency (k, in c) of sugarcane plants as affected by the NH_4^+:NO_3^- ratio in nutrient solution. For example, 80% means a NH_4^+:NO_3^- ratio of 80:20. Plants were supplied with nitrogen at 2 mmol L^{-1} Measurements were taken after 21 days of treatment. Symbols represent the mean value ± se (n=4).

Figure 3. Leaf concentrations of nitrate (a) and ammonium (b), nitrate:ammonium ratio (c) and estimated of leaf N concentration (eLNC, in d) in sugarcane plants as affected by the NH_4^+:NO_3^- ratio in nutrient solution. For example, 80% means a NH_4^+:NO_3^- ratio of 80:20. Plants were supplied with nitrogen at 2 mmol L^{-1}. Measurements were taken after 21 days of treatment. Symbols represent the mean value ± se (n=4).

Figure 4. Leaf content of chlorophyll *a* (a), *b* (b) and *a:b* ratio (c) in sugarcane plants as affected by the NH_4^+:NO_3^- ratio in nutrient solution. For example, 80% means a NH_4^+:NO_3^- ratio of 80:20. Plants were supplied with nitrogen at 2 mmol L^{-1}. Measurements were taken after 21 days of treatment. Symbols represent the mean value ± se (n=4).

Figure 5. Visual aspect (a), dry mass partitioning among roots, culm and leaves (b) and shoot:root dry mass ratio (SDM:RDM, in c) of sugarcane plants under increasing NH_4^+:NO_3^- ratio in nutrient solution. For example, 80% means a NH_4^+:NO_3^- ratio of 80:20. Plants were supplied with nitrogen at 2 mmol L^{-1}. In b, different letters indicate significant differences among treatment means for a given plant organ (Tukey test, $p<0.05$). Photographs and measurements were taken after 21 days of treatment. In b and c, histograms and symbols represent the mean value ± se (n=4).

Fig. 1

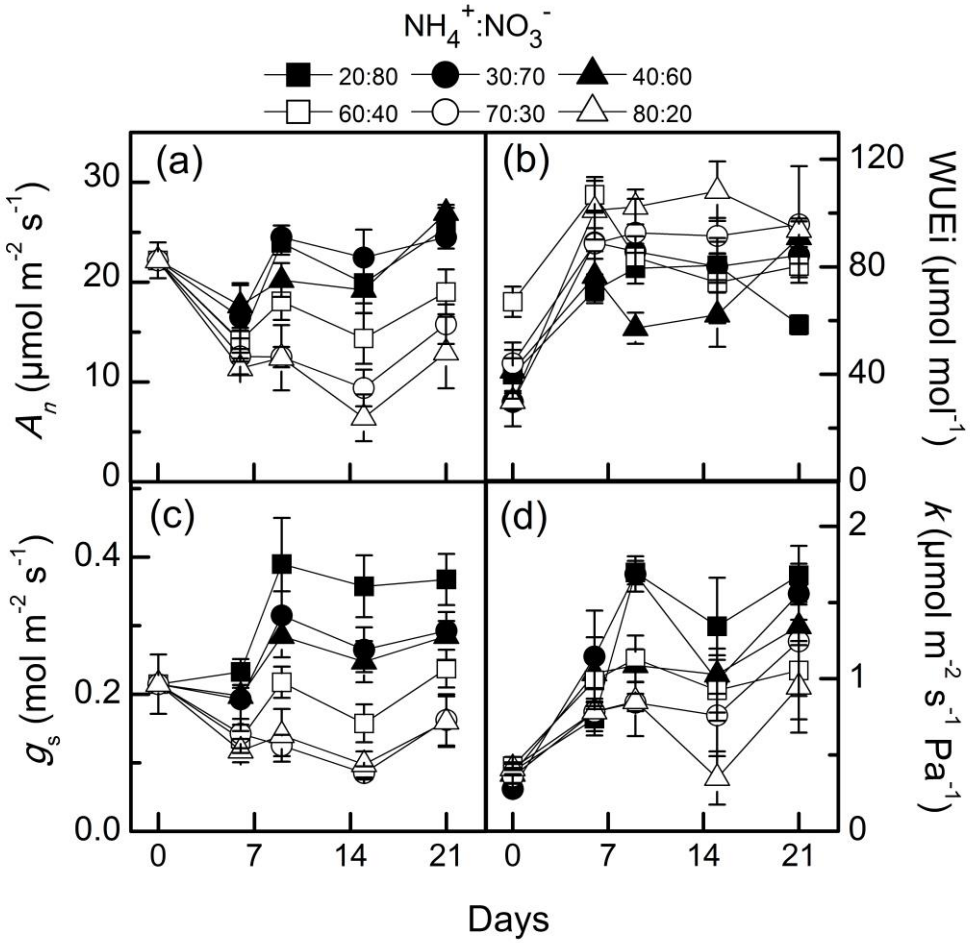

NH4+:NO3-
20:80
30:70
40:60
60:40
70:30
80:20
(a)
(b)
(c)
(d)
An (μmol m-2 s-1)
WUEi (μmol mol-1)
gs (mol m-2 s-1)
k (μmol m-2 s-1 Pa-1)
Days

Fig. 2

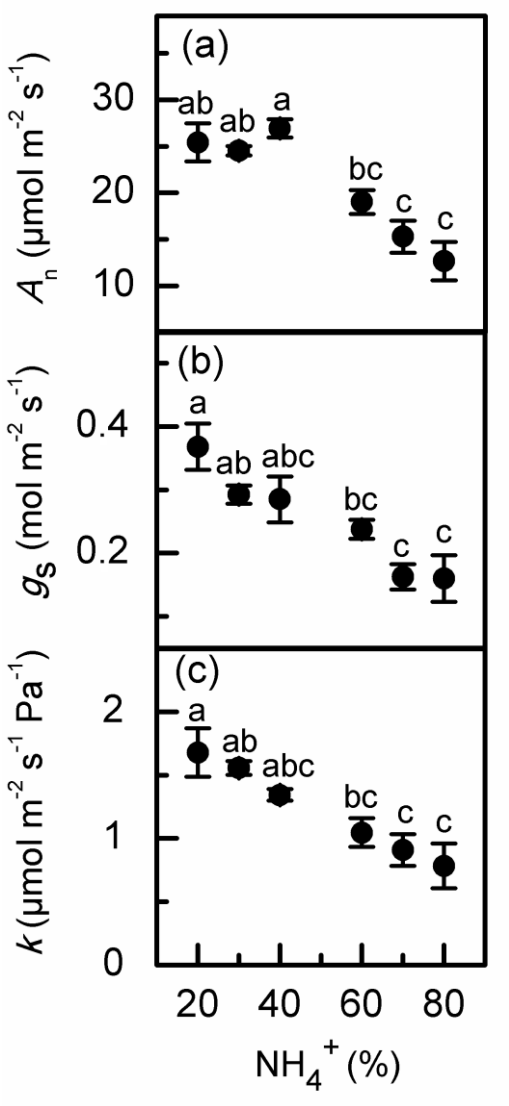

(a)
(b)
(c)
A_n (μmol m^{-2} s^{-1})
g_S (mol m^{-2} s^{-1})
k (μmol m^{-2} s^{-1} Pa^{-1})
ab ab a bc c c
a ab abc bc c c
a ab abc bc c c
30
20
10
0.4
0.2
2
1
0
20 40 60 80
NH_4^+ (%)

Fig. 3

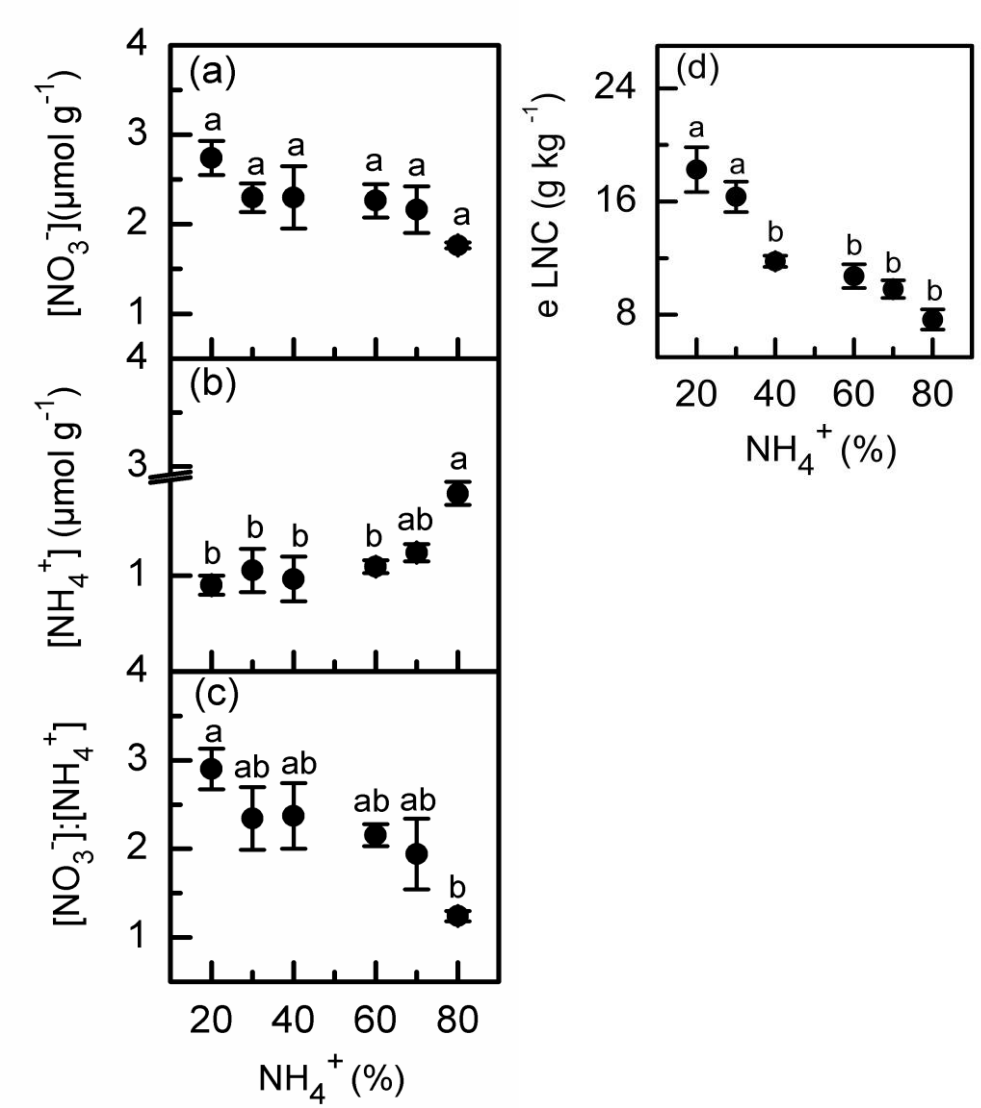


Fig. 4

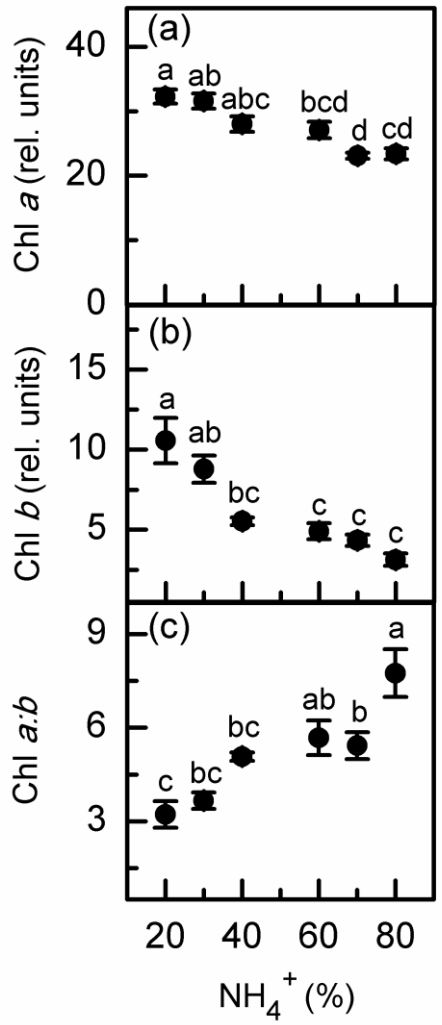
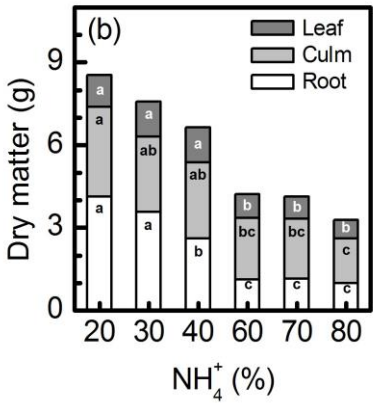
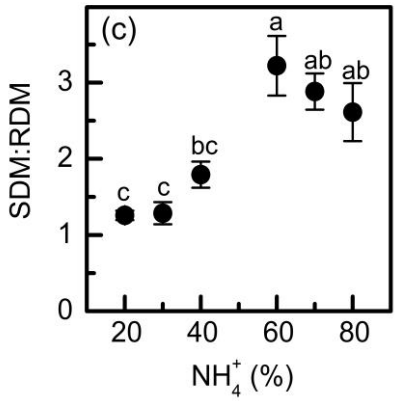

(a)
Chl a (rel. units)
40
20
0
a
ab
abc
bcd
d
cd
(b)
Chl b (rel. units)
15
10
5
a
ab
bc
c
c
c
(c)
Chl a:b
9
6
3
c
bc
bc
ab
b
a
20
40
60
80
NH4+ (%)

Fig. 5


(a)
NH4+ (%)
45 cm
20
30
40
60
70
80


(b)
Leaf
Culm
Root
Dry matter (g)
0
3
6
9
20 30 40 60 70 80
NH4+ (%)


(c)
SDM:RDM
0
1
2
3
c
c
bc
a
ab
ab
20 40 60 80
NH4+ (%)

Capítulo 2

Nitrate supply improves drought tolerance of sugarcane plants through stimulation of nitric oxide production in roots

Nitrate supply improves drought tolerance of sugarcane plants through stimulation of nitric oxide production in roots

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Abstract

Nitric oxide (NO) is an important signaling molecule associated with many biochemical and physiological processes in plants under stressful conditions. One of the ways that plants produce NO is through nitrite (NO_2^-) after nitrate (NO_3^-) reduction. This study aimed to test the hypothesis that plants that receive more NO_3^- as source of nitrogen (N) produce more NO, which reduces the oxidative damage under water deficit and consequently favors photosynthetic metabolism and growth under limiting conditions. Sugarcane plants were grown in nutrient solution with the same amount of N but varying nitrate:ammonium ratio (100:0 and 70:30) and submitted to well-watered conditions (osmotic potential of -0.15 MPa) or water deficit (osmotic potential of -0.75 MPa). In general, plants under water deficit and supplied with more NO_3^- presented higher accumulation of NO_3^- and root NO_2^- than plants supplied with less NO_3^-. Concurrently, these plants also showed higher root nitrate reductase activity and root NO production. In addition, we found higher photosynthetic rates and stomatal conductance in plants supplied with more NO_3^-, which resulted in a higher accumulation of root biomass when compared to those plants receiving less NO_3^-. There was also less accumulation of reactive oxygen species due to increased activity of antioxidant enzymes such as catalase in leaves and superoxide dismutase and ascorbate peroxidase in roots of plants supplied with more NO_3^- and facing water deficit. However, these plants showed higher S-nitrosoglutathione activity in roots, which resulted in less accumulation of S-nitrosothiols in roots. Benefits found in plants supplied with more NO_3^- were offset with cPTIO, a NO scavenger, thus confirming that improvements of leaf gas exchange and plant growth were caused by NO. Our data indicate that increasing NO_3^- supply could be an interesting fertilization strategy for alleviating the negative effects of water deficit on sugarcane plants and then increasing drought tolerance through enhanced NO production.

Keywords: Nitrite, Reactive oxygen species, Reductase Nitrate, Water deficit.

Introduction

Plants are frequently exposed to environmental stresses and water deficit is one of the most important stressors (Ekmekci et al., 2005). Sugarcane plants facing water deficit present reductions in CO_2 assimilation and stomatal opening, resulting in reduced biomass production (Machado et al. 2009; Ribeiro et al. 2013). Stress induced reduction in plant growth may be followed by an alteration in the cellular redox state, leading to oxidative stress and then to the production of reactive oxygen species (ROS) (Saglam et al., 2011; Petrov et al., 2015). In such condition, enzymatic and non-enzymatic antioxidant mechanisms are activated to combat the negative effects of ROS accumulation (Mittler, 2002). Among the antioxidant enzymes involved in plant defense systems, catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) have an essential role in detoxifying cells (Lázaro et al., 2013).

In addition to ROS, reactive nitrogen species (RNS) are produced in response to different environmental situations (Mur, 2013). Nitric oxide (NO) is known as a multifunctional bioactive molecule and plays an important role as a regulator of plant physiology, disease resistance, and stress tolerance (Wang et al., 2010). Increasing evidence indicates NO as a key component of the signaling network, controlling numerous physiological and metabolic processes such as seed germination (Albertos et al., 2015), flowering (He et al., 2004), root growth (Fernandez-Marcos et al., 2011), respiration, stomatal aperture (Wang et al., 2015; Moreau et al., 2010) and adaptive responses to biotic and abiotic stresses (Fatma, 2016; Shan et al., 2015). It has been shown that NO synthesis is increased in plants under drought (Cai et al., 2015; Silveira et al., 2017a). NO and NO-derived molecules play a critical role in intracellular redox signaling and in the activation of antioxidant defense mechanisms (Shi et al., 2014; Hatamzadeh et al., 2015; Silveira et al., 2015). For example, supplying

nitrosogluthatione (GSNO), a NO donor, to sugarcane plants resulted in increased photosynthesis under drought, thereby alleviating the effects of water deficit and promoting plant growth under stressful condition (Silveira et al., 2016). Furthermore, Silveira et al. (2017a) reported that the GSNO supply increased the drought tolerance in sugarcane plants through increases in NO synthesis. The protective effect of the exogenous application of NO donor molecules has been attributed to the elimination of $O_2^{\bullet-}$ and to increases in the activities of antioxidant enzymes in sugarcane under drought (Silveira et al., 2017b). It has also been shown that the application of NO increased the activity of SOD by up to 110% in arsenic-exposed sorghum plants (Saxena and Shekhawat, 2013).

NO can also act as a mediator of ABA-induced stomatal closure to prevent loss of water in plants under drought (Osakabe et al., 2014). The stomatal closure induced by NO and the reduction of transpiration were also demonstrated in wheat and fava plants (García-Mata and Lamattina, 2001; Santisree et al., 2015). Changes in endogenous NO levels and/or exogenous application of NO have shown to regulate resistance to abiotic stress (Ahmad et al., 2018). The mechanisms of NO synthesis and its regulation in plants are complex and poorly understood. On the other hand, NO synthesis in animals has been well documented. NO is bio-synthesized through NO synthase (NOS), which oxidizes arginine to generate citrulline and NO (Alderton et al., 2001). Although some evidence indicate the presence of NOS-like activity in many plant species, genes encoding NOS enzymes have not been identified in land plants (Barroso et al., 1999). In the absence of NOS, one of the most important sources of NO in land plants is through the reduction of nitrite (NO_2^-) by nitrate reductase (NR) (Gupta, 2013; Fancy et al., 2016), a multifunctional enzyme that catalyses nitrate (NO_3^-) reduction to NO_2^- using NADH as an electron donor in the nitrogen assimilation pathway (Katajisto et al. 2015).

Different approaches have led to the proposal that NR is a relevance enzyme for NO production in plants (Gupta, 2013). *Physcomitrella patens* plants treated with sodium tungstate

– a NR inhibitor – had a small NO production compared to the control plants (without the inhibitor), supporting the observation that NO is produced through NR activity (Andrés et al., 2015). Although there is data supporting the relationship between NR and NO production in plants (Mur et al., 2013), some authors have shown that the production of NO by NR represents a small fraction (1-2%) of nitrate reduction then they argue about low efficiency of NO production by NR or unclear relation between NR activity and NO production (Rockel et al., 2002; Yamasaki and Sakihama, 1999). Although the main pathway to produce nitric oxide is not through NR, this enzyme is involved in NO synthesis and therefore controls cellular NO levels (Chamizo-Ampudia et al., 2017).

The cytosolic NO levels depend on the balance between its synthesis and the efficacy of the reactions that lead to NO removal. Once generated, NO can react with other redox-related molecules and potentially regulate protein function. S-nitrosylation is a post-translational modification (PTM) that consists of the reversible attaching of NO to a cysteine residue of a protein leading to the formation S-nitrosothiols (SNOs) (Astier et al., 2012; Fancy et al., 2016) and can regulate the function of the target protein (Corpas et al., 2015). The S-nitrosoglutathione (GSNO), product of the interaction of glutathione (GSH) and NO (Broniowska et al., 2013), is a major nitrosothiol and act as a stable and mobile endogenous reservoir of NO (Leterrier et al., 2011). In this way, GSNO carries NO throughout the plant and provides NO the ability to be a long-distance signaling molecule (Arasimowicz-Jelonek et al., 2014). S-nitrosoglutathione reductase (GSNOR) acts to control GSNO levels, metabolizing it in glutathione disulphide (GSSG) and NH_3 (Ortega-Galisteo et al., 2012). Thus, GSNOR regulates the intracellular levels of GSNO and is important in the maintenance of NO homeostasis (Malik et al., 2011). However, NO derived from NO_3^- assimilation can inhibit GSNOR by S-nitrosylation, avoiding the GSNO degradation. Then, GSNO controls its own

generation and elimination through the modulation of NO_3^- assimilation and the activity of GSNOR (Chamizo-Ampudia et al., 2017; Frungillo et al., 2014).

Here, our aim was to test the hypothesis that sugarcane plants that receive only NO_3^- as source of nitrogen will have higher NR activity and then will produce more NO, compared to plants receiving the same amount of nitrogen but through NO_3^- and NH_4^+ supplying. As consequence of NO production, the oxidative damage will be reduced under water deficit, favoring the photosynthetic metabolism and plant growth.

Materials and Methods

Plant material and growth conditions

Pre-sprouted sugarcane seedlings (*Saccharum* spp.) cv. IACSP95-5000 developed by the Sugarcane Breeding Program of the Agronomic Institute (ProCana, IAC, Brazil) were used. Six-week-old plants with two to three leaves were transferred to plastic boxes (4 L) containing nutrient solution modified of De Armas et al. (1992), containing 5 mmol L^{-1} N (nitrate 90% + ammonium 10%); 9 mmol L^{-1} Ca; 0.5 mmol L^{-1} Mg; 1.2 mmol L^{-1} P; 1.2 mmol L^{-1} S; 24.0 µmol L^{-1} B; 16 µmol L^{-1} Fe; 9 µmol L^{-1} Mn; 3.5 µmol L^{-1} Zn; 1 µmol L^{-1} Cu; and 0.1 µmol L^{-1} Mo. They remained in this solution for twelve days until the establishment of treatments.

The nutrient solution was renewed every three days throughout the experimental period. Electrical conductivity values were maintained between 1.8 and 2.0 mS cm^{-1} and pH kept in 5.9±0.1. The two variables were monitored daily using a portable electrical conductivity meter (mCA 150P, MS Tecnopon Instrumentação, Piracicaba SP, Brazil) and a portable pH meter (mPA 210P, MS Tecnopon Instrumentação, Piracicaba SP, Brazil), respectively. The volume of the nutrient solution was also checked on daily basis and completed with water when

necessary. The nutrient solution was aerated continuously by using an air compressor (Master Super II, Master, São Paulo SP, Brazil).

Plants were grown in growth a chamber, with a 12 h photoperiod, air temperature of 30/20° C (day/night), air relative humidity of 80% and the a photosynthetic photon flux density (PPFD) about 800 µmol m^{-2} s^{-1}.

Experiment I: Inducing NO production under water deficit through nitrate supply

Our previous study has shown that sugarcane plants can be supplied with 30% NH_4^+ in nutrient solution without compromising their photosynthesis and growth (Pissolato et al., 2019). Thus, two NO_3^-:NH_4^+ ratios were chosen to represent the treatments with more and less nitrate: 100:0 and 70:30, while supplying the same amount of nitrogen. An additional factor was water availability and then two osmotic potentials were established: -0.15 MPa (reference, well-hydrated); and -0.75 MPa (water deficit, WD). The WD was induced by the addition of polyethylene glycol (PEG-8000, Fisher Scientific, Leicestershire, UK) to the nutrient solution. To prevent osmotic shock, PEG-8000 was gradually added to the nutrient solution, reducing the osmotic potential of the solution in -0.20 MPa per day, i.e. -0.75 MPa was reached after three days.

All evaluations were taken 24 h after the solution reached the desired osmotic potential. For the biochemical analyses, leaf and root sampling was carried out at the maximum water deficit (7^{th} day) and also at the recovery period (11^{th} day). Samples were collected, immediately immersed in liquid nitrogen and then stored at -80 °C.

Leaf gas exchange

Gas exchange of the first fully expanded leaf with visible ligule was measured daily using an infrared gas analyzer (Li-6400, Licor, Lincoln NE). Leaf CO_2 assimilation (A_n) and stomatal conductance (g_s) were measured under PPFD of 2000 µmol m^{-2} s^{-1} and air CO_2 concentration of 400 µmol mol^{-1}. The measurements were performed between 10:30 and 12:00 h, as done previously (Pissolato et al., 2019). The vapor pressure difference between leaf and air (VPDL) was 2.1±0.2 kPa and leaf temperature was 30±0.4ºC during the evaluations.

Photosynthetic enzymes

The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) was quantified in approximately 200 mg of leaves, which were macerated and homogenized in 100 mM bicine-NaOH buffer (pH 7.8), 1 mM ethylenediaminetetraacetic (EDTA), 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µM leupeptin. The resulting extract was centrifuged at 14.000 *g* for 5 min at 4°C. The initial activity of Rubisco was measured using a reaction medium containing 100 mM bicine-NaOH (pH 8.0) containing 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25 mM NADH, 80 nkat glyceraldehyde-3-phosphate dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase and 80 nkat creatine phosphokinase, and incubated at 25°C. The oxidation of NADH was initiated by adding 0.5 mM ribulose-1,5-bisphosphate (RuBP). A similar aliquot of clarified leaf extract was incubated with the reaction medium for 10 min at 25°C and total Rubisco activity was measured after adding RuBP. The reduction of absorbance at 340 nm was monitored for 3 min (Sage et al., 1988; Reid et al., 1997).

The activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was evaluated in approximately 200 mg of leaves, which were macerated and homogenized in 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM PMSF and centrifuged at 14.000 *g* for

25 min at 4°C. The supernatant was collected and the reaction medium for PEPC activity contained 50 mM Tris-HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 10 mM $NaHCO_3$, 33 nkat malic dehydrogenase and 0.3 mM NADH. The reaction was initiated by adding 4 mM phosphoenolpyruvate at 30°C. The oxidation of NADH was monitored a 340 nm for 1 min (Degl'innocenti et al., 2002).

Proteins were extracted from leaf samples with extraction buffer composed of 100 mM Tris, 1 mM ethylenediaminetetraacetic (EDTA), 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and separated by SDS-PAGE (Laemmli, 1970). The first gel was stained with Comassie Brilliant Blue and the second was used for Western blot. Denatured proteins were transferred to nitrocellulose membrane (Towbin et al., 1979) and detection of the polypeptide was performed using antibodies against PEPC and Rubisco. The procedure was performed according to the manufacturer's manual (Agrisera). SDS-PAGE electrophoresis was performed with equal amounts of protein per lane.

Leaf relative water content (RWC)

The relative water content was calculated using the fresh (FW), turgid (TW) and dry (DW) weights of leaf discs according to Jamaux et al. (1997): $RWC=100\times[(FW-DW)/(TW-DW)]$.

Reactive oxygen species

The concentration of the superoxide anion ($O_2^{\bullet-}$) was determined in 50 mg of samples incubated in an extraction medium consisting of 100 μM EDTA, 20 μM NADH, and 20 mM sodium phosphate buffer, pH 7.8 (Kuo and Kao, 2003). The reaction was initiated by adding

25.2 mM epinephrine in 0.1 N HCl. The samples were incubated at 28°C under stirring for 5 min and the absorbance was read at 480 nm for 5 min. $O_2^{\bullet -}$ production was assessed by the accumulation of adrenochrome using a molar extinction coefficient of 4.0×10^3 M^{-1} cm^{-1} (Gay and Gebicki, 2000).

The quantification of hydrogen peroxide (H_2O_2) was performed following the method of Alexieva et al. (2001). Homogenates were obtained from 100 mg of fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and 0.1% of trichloroacetic acid (TCA) solution (w/v). The extract was centrifuged at 10.000 *g* and 4°C for 15 min. The reaction medium consisted in 1 mM KI, 0.1 M potassium phosphate buffer (pH 7.5) and crude extract. The microtubes were incubated on ice under dark for 1 h. After this period, the absorbance was read at 390 nm. A standard curve was obtained with H_2O_2 and the results were expressed as µmol H_2O_2 g^{-1} FW.

Lipid peroxidation

The concentration of malondialdehyde (MDA) was measured and used as a proxy of lipid peroxidation. Plant tissue (200 mg) was macerated in extraction medium containing 0.1% TCA (w/v) and centrifuged at 10.000 *g* for 15 min. The supernatant was added in 0.5% thiobarbituric acid (w/v) in 20% TCA (w/v), and the mixture was incubated at 95°C. Two hours after the reaction, the absorbance was measured at 532 and 600 nm. The absorbance at 600 nm was subtracted from the absorbance at 532 nm and the MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Protein extraction and antioxidant activity

Plant extracts were obtained from the macerate of 100 mg of fresh tissue (roots or leaves) with liquid nitrogen, 1% PVPP and extraction medium containing 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM phenylmethylsulfonic fluoride (PMSF). After centrifugation of the homogenates at 15.000 *g* for 15 min and 4°C, supernatants (crude extracts) were collected and preserved on ice. The protein content of the enzymatic extracts were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

The crude enzymatic extracts for the determination of superoxide dismutase activity (SOD), catalase (CAT) and ascorbate peroxidase (APX) were obtained by maceration of 100 mg of plant tissue in specific medium, followed by centrifugation at 12.000 *g* for 15 min at 4°C. The specific medium for CAT and SOD consisted of 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM PMSF and 1% polyvinylpolypyrrolidone (PVPP), following Peixoto et al. (1999). The specific medium for APX was composed by 50 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid and 1 mM EDTA (Nakano and Asada, 1981).

The superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). The crude extract was added to the reaction medium consisting of 100 mM sodium phosphate buffer (pH 7.8), 50 mM methionine, 5 mM EDTA, deionized water, 100 μM riboflavin and 1 mM nitro blue tetrazolium chloride (NBT). A group of tubes was exposed to light (fluorescent lamp, 30 W) for 10 min, and another group remained in darkness. The absorbance was measured at 560 nm and one unit of SOD is the amount of enzyme required to inhibit the NBT photoreduction in 50%, being expressed as U min^{-1} mg^{-1} of protein.

The catalase (CAT, EC 1.11.1.6) activity was quantified following the procedure described in Havir and McHale (1987). The crude extract was added to the reaction medium composed by 100 mM potassium phosphate buffer (pH 6.8), deionized water and 125 mM

H_2O_2. The reaction was carried out in a water bath at 25°C for 2 min and CAT activity was assessed by the decrease in absorbance at 240 nm, using the molar extinction coefficient of 36 M^{-1} cm^{-1} (Anderson et al., 1995) and expressed as nmol min^{-1} mg^{-1} of protein.

The ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated as described by Nakano and Asada (1981). The crude extract was added to the reaction medium consisting of 100 mM potassium phosphate buffer (pH 6.8), deionized water, 10 mM ascorbic acid and 10 mM H_2O_2. The reaction was carried out in a water bath at 25°C for 2 min and APX activity monitored by the decrease in absorbance at 290 nm, using the molar extinction coefficient of 2.8 M^{-1} cm^{-1} and expressing as µmol min^{-1} mg^{-1} of protein.

Nitrate, nitrite and ammonium

Fresh leaf samples (500 mg) were ground in liquid nitrogen and 2.5 mL of extraction medium containing methanol:chloroform:water (12:5:3 v/v) added to the powder. After centrifugation at 2000 *g* for 5 min, the supernatants were collected and 1 mL of chloroform and 1.5 mL of water were added to 4 mL of the supernatant. The extract was shaken vigorously and then centrifuged again for 3 min at 2000 *g* for phase separation. The upper aqueous phase was collected and maintained in a water bath at 37 °C for two hours to remove traces of chloroform and then the extracts were stored at -20 ºC (Bieleski and Turner, 1966).

For nitrate determination, an aliquot (50 µL) of the extract was pipetted into test tubes containing 0.2 mL of reaction medium (5% w/v salicylic acid + concentrated sulfuric acid). After 20 min, 4.75 mL of 2N NaOH was added and the solution stirred. After cooling at room temperature, the absorbance was read in a spectrophotometer (Ultrospec 1000, Pharmacia Biotech, Cambridge, England) at 410 nm and the nitrate content was calculated from a standard curve using KNO_3 (100-1000 nmol) (Cataldo et al., 1975). For nitrite determination, an aliquot

(1 mL) of the extract was added in 0.3 mL of 1% sulfanilamide solution in 3N HCl and 0.3 mL of 0.02% n-naphthyl diamine solution. The tubes were allowed to stand for 30 min in the dark and room temperature. Then, 0.9 mL of deionized water was added and nitrite content quantified after reading absorbance in a spectrophotometer at 540 nm (Hageman and Reed, 1980). For ammonium content, an aliquot (50 µL) the extract was added to microtubes. Then, 250 µL of solution A (1% phenol + 0.005% sodium nitroprusside) were added followed by 250 µL of solution B (0.5% sodium hydroxide containing 2.52% sodium hypochlorite). The tubes were kept for 35 min in a water bath at 37 °C and the absorbance read at 625 nm after cooling to room temperature (McCullough, 1967). A standard curve of $(NH_4)_2SO_4$ (0-100 nmol N) was used to estimate the ammonium content.

Nitrate reductase (NR) activity

Leaf and root NR activity was estimated as the rate of nitrite (NO_2^-) production (Cambraia et al., 1989). The enzyme extract was obtained by macerating 200 mg of fresh tissue with liquid nitrogen followed by homogenization with extraction medium containing 0.1 M tris-HCl buffer (pH 8.1), 4 mM $NiSO_4$, 20 mM reduced glutathione (GSH), deionized water and 0.5 mM PMSF. Then, the crude extracts were centrifuged at 10.000 *g* for 10 min at 4°C and the supernatant was collected and maintained on ice. The extract was added in reaction medium containing 100 mM Tris-HCl buffer (pH 7.5), 10 mM KNO_3, 0.05 mM NADH and triton X-100 1%, mixed and incubated at 30°C for 10 min. The reaction was quenched by adding 1% sulfanilamide in 0.01% N-naphthyl ethylenediamine and 1M hydrochloric acid (HCl). Nitrite production was determined by absorbance at 540 nm. The values obtained were compared to those of a standard curve using KNO_2 (0 to 100 µM), and normalized against protein content.

S-nitrosogluthatione reductase (GSNOR) activity

Leaf and root GSNOR activity was determined spectrophotometrically at 25°C by monitoring the oxidation of NADH at 340 nm (Rodríguez-Ruiz, et al. 2017, adapted). Briefly, 200 mg of fresh tissue were ground with liquid nitrogen, resuspended in 20 mM HEPES buffer (pH 8.0), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonic fluoride (PMSF) and centrifuged for 10 min at 10.000 *g* and 4ºC. The enzyme extract was added in reaction medium consisting of 20 mM HEPES buffer (pH 8.0) and 1.8 mM NADH at 25 ºC, and maintained in the dark. The reaction was started by adding 4 mM GSNO (Silveira et al., 2016) and the GSNOR activity was estimated using the NADH molar extinction coefficient (E_{340nm} 6.22 mM^{-1} cm^{-1}) and normalized by the protein content. The GSNOR activity was expressed as nmol NADH min^{-1} mg^{-1} protein.

S-nitrosothiols

The total leaf and root proteins were extracted in deionized water and the resulting homogenate was used to estimate the S-nitrosothiol content through an amperometer, as described by Santos et al. (2016) and Zhang et al (2000). Measurements were performed with the WPI amperometer TBR 4100/1025 (World Precision Instruments Inc., Sarasota, FL, USA) and a specific nitric oxide sensor, ISO-NOP (2 mm). Aliquots of aqueous suspension were added to the sample compartment containing aqueous copper chloride solution (0.1 mol L^{-1}). This condition allowed the detection of free NO released from the S-nitrosothiols present in the leaf and root protein homogenate. The samples were run in triplicate and the calibration curve was obtained with newly prepared GSNO solutions. The data were compared with the standard curve obtained and normalized against fresh weight.

Intracellular NO detection

Confocal laser scanning microscopy (CLSM) method using fluorescence probes are common in NO research. From the family of diaminofluoresceins (DAF), 4,5-diaminofluorescein diacetate (DAF-2DA) is preferred because it enters the cell and reacts with NO to produce the fluorescent triazole DAF-2T (Kojima et al., 1998) allowing the identification and localization of NO inside cells.

Fresh leaf and root segments were incubated in MES-KCl buffer (10 mM MES, 50 mM KCl, 0.1 mM $CaCl_2$, pH 6.15), at room temperature for 15 min. Then, these segments were incubated in a solution of 4,5-diaminofluorescein diacetate (DAF2-DA) 10 µM, mixing per 40 min in dark at room temperature (Desikan et al., 2002; Bright et al., 2009). The samples were washed with buffer to remove the excess of DAF2-DA, placed onto a glass slide and covered with a glass slip before observing fluorescence using inverted confocal microscopy with excitation at 488 nm and emission at 515 nm (Model Zeiss LSM510, Carl Zeiss AG, Germany). Photos were taken with a 10x magnification, 15 s exposure and 1x gain. Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA) and data are presented as mean pixel intensities.

Biometry

Leaf and root dry biomasses were quantified after drying samples in an oven (60 °C) with forced-air circulation until constant weight. In addition, the leaf area was evaluated with a portable leaf area meter (model LI-3000, Li-Cor Inc., Lincoln NE, USA).

Experiment II: Using cPTIO to offset the benefits of NO in plants under water deficit

An extra experiment was performed to verify if the benefits found in plants supplied with 100% nitrate and subjected to water deficit were in fact caused by NO. We used a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), which is a stable organic radical developed by Akaike and Maeda (1996) and has been widely used as a control since it oxidizes the NO molecule to form NO_2. In plants supplied with 100% NO_3^- as N source, the following treatments were evaluated: (a) well-watered condition, with the osmotic potential of nutrient solution of -0.15 MPa; (b) water deficit, with the osmotic potential of nutrient solution of -0.75 MPa; and (c) same as b with cPTIO 100 µM supply.

Firstly, plants were removed and roots placed in a moist chamber, where they were sprayed with cPTIO and remained in dark for 1 hour. After, we returned the plants to the boxes with the original nutrient solution. This procedure was performed for four consecutive days from the moment the water deficit (-0.75 MPa) was installed and there was a reduction of photosynthesis. In addition, we evaluated the production of intracellular NO and plant biomass as described previously.

Experimental design and statistical analyses

The experimental design was completely randomized and two causes of variation (factors) were analyzed: water availability and nitrogen source availability. Data were then subjected to the analysis of variance (ANOVA) and when statistical significance was detected, the mean values (n=4) were compared by the Tukey test (p<0.05) using the software Assistat version 7.7 (UFCG, Campina Grande PB, Brazil).

Results

Water deficit (WD) induced a large reduction in leaf CO_2 assimilation (A_n) of sugarcane plants, however, plants that received more NO_3^- (100:0) had higher photosynthetic rates than plants that received less NO_3^- (70:30) (Fig. 1a). In addition, these plants showed a faster recovery of A_n compared to the plants under 70:30 supply (Fig. 1a). The same results were observed for stomatal conductance (g_s) (Fig. 1b). The content of Rubisco was decreased in both treatments under WD and its activity as well (Fig. 2b,d). We did not notice significant changes between treatments for the content and activity of PEPC at the maximum water deficit (Fig. 2a,d). During the recovery, PEPC activity was reduced in plants that faced water deficit (Fig. 2a).

Leaf nitrate concentration [NO_3^-] was higher in plants under WD than in well-hydrated plants (reference), but no difference was found due to nitrate supply under WD (Fig. 3a). However, root [NO_3^-] was considerably higher in plants supplied with more NO_3^- and subjected to WD (Fig. 3b). The concentration of nitrite [NO_2^-] in leaves did not vary significantly between treatments (Fig. 3c). We found higher NO_2^- accumulation in roots supplied with 100:0 solution as compared to 70:30 under WD (Fig. 3d). We did not find differences in [NH_4^+] between treatments under WD, regardless the plant organ (Fig. 3e,f). No differences were observed in leaf and root [NO_3^-], [NO_2^-] and [NH_4^+] during the recovery period (Fig. 3).

Nitrate reductase (NR) activity was higher in both leaves and roots of plants under WD and supplied with 100:0 solution than those supplied with 70:30 (Fig. 4a,b). In the recovery period, we did not observe differences between treatments for leaf NR activity. On the other hand, in the roots NR activity was higher in treatments under water deficit (Fig. 4b).

At the maximum water deficit, we found a higher SOD activity in roots of plants that received 100% of nitrogen as NO_3^- in comparison to the other treatments (Fig. 5b), but we did

not observe differences in leaf SOD activity among treatments under water deficit (Fig. 5a). We did not find significant differences in root CAT activity between treatments, but higher leaf CAT and root APX activities were found in plants supplied with more NO_3^- under WD (Fig. 5d,e).

Under water deficit, plants supplied with 70:30 solution presented higher accumulation of $O_2^{\bullet-}$ in leaves compared to the plants supplied with 100:00 solution (Fig. 6a). In addition, a higher H_2O_2 accumulation was found in roots of plants receiving 70:30 solution as compared to ones under 100:0 supplying (Fig. 6d). Although the treatment with 70:30 solution caused higher accumulation of $O_2^{\bullet-}$ (Fig. 6a) and H_2O_2 (Fig. 6d) in leaves and roots, respectively, we did not find differences in MDA content (Fig. 6e,f).

The intracellular NO in roots of plants under WD and that received more NO_3^- was considerably higher than in plants that received 70:30 solution (Fig. 7b). Such a difference was not observed in leaves (Fig. 7a) and intracellular NO production was very low in reference plants (Fig. 7). Increases in root SNO content were found in plants receiving 70:30 solution and subjected to WD (Fig. 8d). We did not find differences in leaf SNO concentration between treatments either under WD or during the recovery (Fig. 8c,d). On the other hand, plants under WD supplied with more NO_3^- showed higher root GSNOR activity when compared to plants supplied with less NO_3^- (Fig. 8b).

The root dry mass of plants supplied with less NO_3^- was significantly reduced under WD (Fig. 9b). In addition, the lowest absolute values of shoot dry mass and leaf area index were also found in plants supplied with less NO_3^- under WD (Fig. 9a,c).

The NO scavenger cPTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) was sprayed on roots of plants under WD and supplied with 100% NO_3^- as source of nitrogen. The production of intracellular NO was reduced both in leaves and roots of plants treated with cPTIO (Fig. 10a,b) and then plants receiving cPTIO showed lower photosynthetic rates and

stomatal conductance as compared to cPTIO free plants under WD (Fig. 11a,b). We also observed a reduction of root dry mass of plants under WD supplied with 100% NO_3^- and cPTIO (Fig. 12b).

Discussion

Nitrate supply stimulates root NO production, improving photosynthesis and antioxidant metabolism

Our findings suggest that the nitrate reductase (NR) is an important enzymatic pathway for nitric oxide (NO) synthesis and sugarcane plants supplied with more NO_3^- presented enhancement of drought tolerance. Here, we observed higher NO_3^- accumulation in roots of plants under water deficit that received more NO_3^- as source of nitrogen (Fig. 3b), and consequently, presented higher NO_2^- production (Fig. 3d), when compared to roots of plants under water deficit that received less NO_3^- (Fig. 3b,d). Such findings are in agreement with higher root NR activity (Fig. 4), which reduces NO_3^- to NO_2^- in the nitrogen assimilation pathway (Katajisto et al., 2015). In addition to its ability to reduce NO_3^- to NO_2^-, NR may also reduce NO_2^- to NO (Fancy et al., 2016). In fact, the highest root production of intracellular NO was found in plants under water deficit that received more NO_3^- (Fig. 7b). In *Physcomitrella patens*, decreases in NR activity were associated with drastic reductions in NO production, another evidence that NR is an important pathway for NO production in plants (Andrés et al., 2015). It is worth noting that under ideal growth conditions we found low NO production even in plants supplied only with nitrate (Fig. 7). However, increases in NO production are expected under stressful conditions due to the accumulation of NO_2^- (Mur et al., 2012), as found here in plants fed only NO_3^- (Fig. 3d).

In the last decades, rapidly increasing evidence has indicated that NO is a key player in plant response to many stressful conditions. NO is considered to play a role in stress-induced oxidative defense in plants. During cell detoxification, $O_2^{\bullet-}$ produced in the mitochondria, chloroplasts and peroxisomes is dismuted to H_2O_2 by superoxide dismutase (SOD), which is rapidly eliminated by the catalase (CAT) and ascorbate peroxidase (APX), producing H_2O and O_2 (Lázaro et al., 2013). Here, we observed a higher SOD activity in roots of plants under water deficit that received more NO_3^- (Fig. 5b), but root $O_2^{\bullet-}$ concentration remained similar in all treatments (Fig. 6b). Interestingly, we observed a lower accumulation of $O_2^{\bullet-}$ in leaves of plants under water deficit supplied with only NO_3^- (Fig. 6a), where SOD activity remained similar to one found in leaves of plants supplied with NO_3^- and NH_4^+ (Fig. 5a). As an alternative explanation, our data suggest that reductions of leaf $O_2^{\bullet-}$ level in plants supplied with 100% NO_3^- are related to the interaction of this radical with NO, which generates peroxynitrite ($ONOO^-$), which may add a nitro group to tyrosine residues, a process known as tyrosine nitration (Wullf et al., 2009). Although tyrosine nitration was originally considered as an indicative of stress, recent evidence suggests its involvement in cell signaling (Mengel et al., 2013).

Root H_2O_2 concentration was lower in plants under water deficit that received more NO_3^- than in plants under similar conditions that received less NO_3^- (Fig. 6d), indicating an efficient detoxification through increased root APX activity (Fig. 5d). In fact, the activation of antioxidant mechanisms to maintain ROS homeostasis often involves the participation of NO (Hatamzadeh et al., 2015; Silveira et al., 2015). Many reports show that exogenous NO application improves abiotic stress tolerance, causing decreases in H_2O_2 and MDA levels (Gross et al., 2013). Exogenous application of NO inhibits ROS accumulation in many plant species under stress conditions (Verma et al., 2013). In cucumber and rice, the exogenous application of NO increased the drought tolerance due to increase of antioxidants and

elimination of ROS (Farooq et al., 2009). Sugarcane plants supplied with GSNO, a NO donor, showed increases in the activity of antioxidant enzymes, such as SOD in leaves and CAT in roots, indicating a higher antioxidant capacity under water deficit (Silveira et al., 2017b).

The increased root SOD and APX activities in plants under water deficit and supplied with more NO_3^- (Fig. 5b,d) may be due to S-nitrosylation processes induced by NO. In pea (*Pisum sativum*), S-nitrosylation increased the activity of cytosolic APX (Begara-Morales et al., 2014). However, we observed higher levels of S-nitrosothiols (SNOs) in roots of plants under water deficit that received less NO_3^- (Fig. 8d). At this point, one should consider that NO-mediated post-translational modifications on target proteins may be positive or negative (Nabi et al, 2019). According to Clark et al. (2000), S-nitrosylation can inhibit CAT activity, which implies that the reduced level of S-nitrosylation can increase CAT activity during metal stress, thus increasing ROS detoxification. In this way, we may argue that the higher SNOs concentration found in plants that received less nitrate (Fig. 8d) is associated with changes in antioxidant system that lead to increases of $O_2^{\bullet-}$ concentration in leaves and H_2O_2 in roots (Fig. 6a,d). It has been proposed that S-nitrosylation can regulate the H_2O_2 level, controlling both the antioxidant defense system and the ROS-producing enzymes (Ortega-Galisteo et al., 2012). Here, we observed lower SNOs accumulation and higher GSNOR activity in roots of plants under water deficit that received more NO_3^- when compared to plants under water deficit that received less NO_3^- (Fig. 8b,d).

GSNOR enzyme can break down S-nitrosoglutathione (GSNO), a SNO, reducing GSNO levels and consequently decreasing the total cellular level of S-nitrosylation (Feechan et al., 2005). Thus, it indirectly controls the overall SNOs within cells (Feechan et al., 2005), suggesting that GSNOR could be crucial in regulating the pool of total SNOs. Increase of GSNOR activity was found in pea plants under salt stress, which contributed to the reduction of S-nitrosylation (Camejo et al., 2013). As GSNO is an NO donor, we can suggest that increase

of root GSNOR activity in plants under water deficit that received more NO_3^- (Fig. 8b) is related to the reduction of GSNO levels due to the high synthesis of NO in roots (Fig. 7b). In fact, high levels of reactive nitrogen species (RNS) may be harmful to plants (Nabi et al., 2019) and the absence of GSNOR activity in plants results in a significant increase in SNOs levels and impairment of plant immunity (Feechan et al., 2005), plant growth and development (Kwon et al., 2012). Gong et al. (2015) demonstrated that absence of GSNOR activity increased the sensitivity of tomato plants (*Solanum lycopersicum*) to alkaline stress due to the excessive accumulation of NO and SNOs, causing higher level of endogenous S-nitrosylation and turning plants insensitive to the ABA-induced stomatal closure.

Stomatal closure is the primary response of plants submitted to water deficit, which reduces the availability of CO_2 for photosynthesis and then decreases biomass production (Machado et al. 2009; Ribeiro et al. 2013). Although water deficit reduced the photosynthetic rates in sugarcane, we observed an improvement in leaf CO_2 assimilation and stomatal conductance in plants under water deficit that received more NO_3^- in relation to those that received less NO_3^- (Fig. 1a,b). As for biomass, plants under water deficit and receiving less NO_3^- presented lower accumulation of root biomass as compared to the plants that received more NO_3^- and were under water deficit. In fact, these latter plants and reference plants presented similar root biomass (Fig. 9b). This higher accumulation of root biomass found in plants under water deficit that received more NO_3^- may be associated with intracellular NO content (Fig. 7b), as found by Silveira et al. (2017). The root system is able to perceive the reductions in water availability and to produce chemical signals that regulate the flow of water from roots to shoots (Tardieu, 1996). NO is one of those chemical signals and plays a crucial role in stimulating the expansion and development of the root system (Xu et al., 2017; Silveira et al., 2016). In addition, NO has been shown to alter gene expression in response to water deficit. Transgenic *A. thaliana* expressing the NOS enzyme submitted to drought, presented

transcriptional alterations in genes involved in redox metabolism and sugar metabolism. These transcriptional changes were accompanied by higher survival rates and higher biomass production, indicating a protective effect of NO (Shi et al., 2014). In fact, NO is involved in signaling cascades that lead to the formation of root hairs in *A. thaliana* (Lombardo et al., 2012) and increases of root biomass in sugarcane (Silveira et al., 2016). The main function of the root hair is to increase the root surface and improve the absorption of water and nutrients. In this context, increases in NO content can trigger root formation and improve water uptake, reducing the impact of low water availability on leaf water status and allowing higher stomatal conductance, as noticed herein and also by Silveira et al. (2017).

Are those beneficial effects on leaf gas exchange due to NO production?

Herein, we used 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), an endogenous NO scavenger (García-Mata et al., 2002) to prove the effects of NO on leaf gas exchange and plant growth. cPTIO drastically reduced the DAF-2DA in plants under water deficit, indicating lower accumulation of NO both in leaves and roots (Fig. 10a,b). As consequence, plants showed even lower stomatal conductance and photosynthesis when compared to plants under water deficit and not supplied with cPTIO (Fig. 11a,b). In addition, cPTIO sprays also reduced root biomass (Fig. 12b), as found previously (Fig. 9b). Taken together, our data clearly show that the positive effects observed here in plants under water deficit that received only nitrate is due to the increased NO production.

Conclusion

Sugarcane plants grown in nutrient solution containing more nitrate were more tolerant to water deficit and this response was associated with increased NO production and higher nitrate reductase activity, mainly in roots. This indicates that NO synthesis through nitrate reductase could be an important pathway in sugarcane under water deficit. Here, only 30% more nitrate was enough to increase NO production and alleviate the effects of water deficit in sugarcane by increasing the activity of antioxidant enzymes, improving photosynthetic CO_2 assimilation, stomatal conductance and root growth. From a broad perspective, our data show that small changes in proportion of NO_3^-:NH_4^+ in nitrogen management may be beneficial to sugarcane plants. Such hypothesis must be tested under field conditions, where interactions among nutrients, soil-root interactions and soil nitrogen dynamics are important aspects.

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Figures captions

Figure 1. Leaf CO_2 assimilation (A_n, in a), stomatal conductance (g_S, in b) and leaf relative water content (RWC, in c) in sugarcane plants maintained hydrated (ref, white symbols) and subjected to water deficit (WD, black symbols) and supplied with different NO_3^-:NH_4^+ ratios: 100:00 and 70:30. The white area indicates the period of maximum water deficit and the shaded area indicates the period of recovery. Symbols represent the mean value of four replications ± se. Asterisks indicate significant differences between treatments under water deficit (Tukey test, $P<0.05$).

Figure 2. *In vitro* activity of phosphoenolpyruvate carboxylase (PEPC, in a) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, in b), and immunoblots of total leaf proteins probed with antisera raised against PEPC (c) and Rubisco (d) and their relative abundances in sugarcane plants maintained hydrated (reference, white bars) and subjected to water deficit (WD, gray bars) and supplied with different NO_3^-:NH_4^+ rates: 100:00 and 70:30. The white area indicates the period of maximum water deficit and the shaded area indicates the period of recovery. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 3. Concentration of nitrate ($[NO_3^-]$ in a and b), nitrite ($[NO_2^-]$ in c and d) and ammonium ($[NH_4^+]$ in e and f), in leaves (a, c and e) and roots (b, d and f) of sugarcane plants maintained hydrated (reference, white bars) and subjected to water deficit (WD, gray bars) and supplied with different NO_3^-:NH_4^+ rates: 100:00 and 70:30. The white area indicates the period of maximum water deficit and the shaded area indicates the period of recovery. Symbols and bars

represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 4. Activity of leaf (a) and root (b) nitrate reductase in sugarcane plants maintained hydrated (reference, white bars) and subjected to water deficit (WD, gray bars) and supplied with different NO_3^-:NH_4^+ rates: 100:00 and 70:30. The white area indicates the period of maximum water deficit and the shaded area indicates the period of recovery. Symbols and bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 5. Activity of superoxide dismutase (SOD, in a and b), ascorbate peroxidase (APX, in c and d) and catalase (CAT, in e and f) in leaves (a, c and e) and roots (b, d and f) of sugarcane plants maintained hydrated (reference, white bars) and subjected to water deficit (WD, gray bars) and supplied with different NO_3^-:NH_4^+ rates: 100:00 and 70:30. The white area indicates the period of maximum water deficit and the shaded area indicates the period of recovery. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 6. Concentration of superoxide anion ($O_2^{\bullet -}$, in a and b), hydrogen peroxide (H_2O_2, in c and d) and malondialdehyde (MDA in e and f) in leaves (a, c and e) and roots (b, d and f) of sugarcane plants maintained hydrated (reference, white bars) and subjected to water deficit (WD, gray bars) and supplied with different NO_3^-:NH_4^+ rates: 100:00 and 70:30. The white area indicates the period of maximum water deficit and the shaded area indicates the period of recovery. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 7. Confocal microscopy images demonstrating intracellular NO synthesis in leaves (a) and roots (b) and mean pixel intensity by ImageJ in sugarcane leaves maintained hydrated (reference, white bars) and subjected to water deficit (WD, gray bars) and supplied with different NO_3^-:NH_4^+ rates: 100:00 and 70:30. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 8. S-nitrosoglutathione reductase activity (GSNOR, in a and b) and S-nitrosothiol content (SNO, in c and d) in leaves (a and c) and roots (b and d) of sugarcane plants maintained hydrated (reference, white bars) and subjected to water deficit (WD, gray bars) and supplied with different NO_3^-:NH_4^+ rates: 100:00 and 70:30. The white area indicates the period of maximum water deficit and the shaded area indicates the period of recovery. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 9. Shoot (a) and root (b) dry mass and leaf area (c) of sugarcane plants maintained hydrated (reference, white bars) and subjected to water deficit (WD, gray bars) and supplied with different NO_3^-:NH_4^+ rates: 100:00 and 70:30. Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 10. Confocal microscopy images demonstrating intracellular NO synthesis in leaves (a) and roots (b) of sugarcane plants maintained hydrated (reference, white bar), subjected to water deficit (WD, gray bar) and subjected to water deficit + cPTIO (gray striped bar) and supplied with only NO_3^- as a source of N (100%). Mean pixel intensities are also shown. Bars represent

the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 11. Leaf CO_2 assimilation (A_n, in a), stomatal conductance (g_S, in b) in sugarcane plants maintained hydrated (ref, white symbol), subjected to water deficit (WD, black symbol) and subjected to water deficit + cPTIO (white symbol with X in the center) and supplied with only NO_3^- as a source of N (100%). Asterisks indicate significant differences between treatments under water deficit (Tukey test, $P<0.05$).

Figure 12. Shoot (a) and root (b) dry mass, and leaf area (c) of sugarcane plants maintained hydrated (reference, white bar), subjected to water deficit (WD, gray bar) and subjected to water deficit + cPTIO (gray striped bar) and supplied with only NO_3^- as a source of N (100%). Bars represent the mean value of four replications ± se. Different letters indicate statistical difference among treatments (Tukey test, $p<0.05$).

Figure 13. Visual aspect of sugarcane plants at the end of the experiment. Ref: plants maintained hydrated; WD: plants subjected to water deficit; WD + cPTIO: plants subjected to water deficit and sprayed with. All treatments were supplied with only NO_3^- as a source of N (100%).

Fig. 1

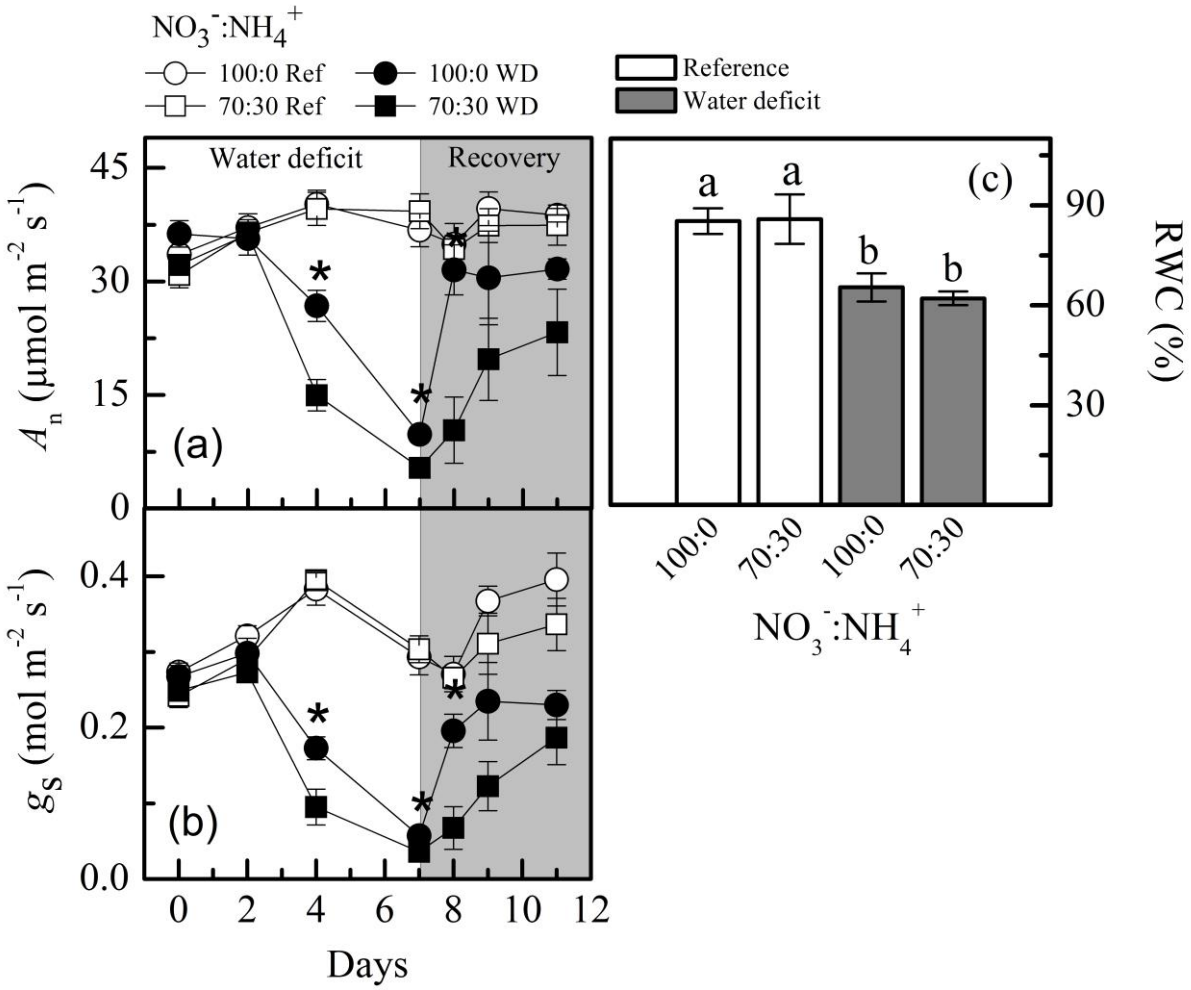
NO3-:NH4+
100:0 Ref
70:30 Ref
100:0 WD
70:30 WD
Reference
Water deficit
Water deficit
Recovery
(a)
(b)
(c)
An (µmol m-2 s-1)
gs (mol m-2 s-1)
RWC (%)
Days
100:0
70:30
100:0
70:30
NO3-:NH4+

Fig. 2

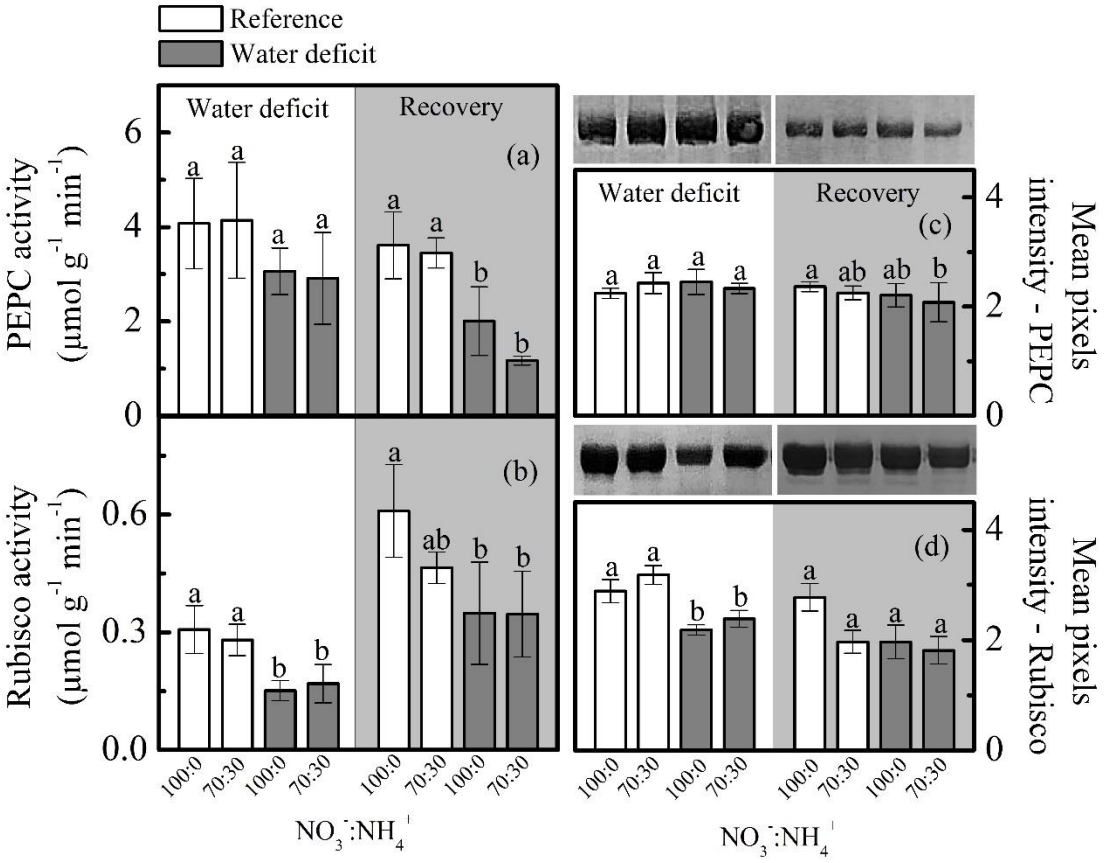
Reference
Water deficit
Water deficit
Recovery
(a)
(b)
(c)
(d)
PEPC activity (μmol g-1 min-1)
Rubisco activity (μmol g-1 min-1)
Mean pixels intensity - PEPC
Mean pixels intensity - Rubisco
100:0
70:30
NO3-:NH4+

Fig. 3

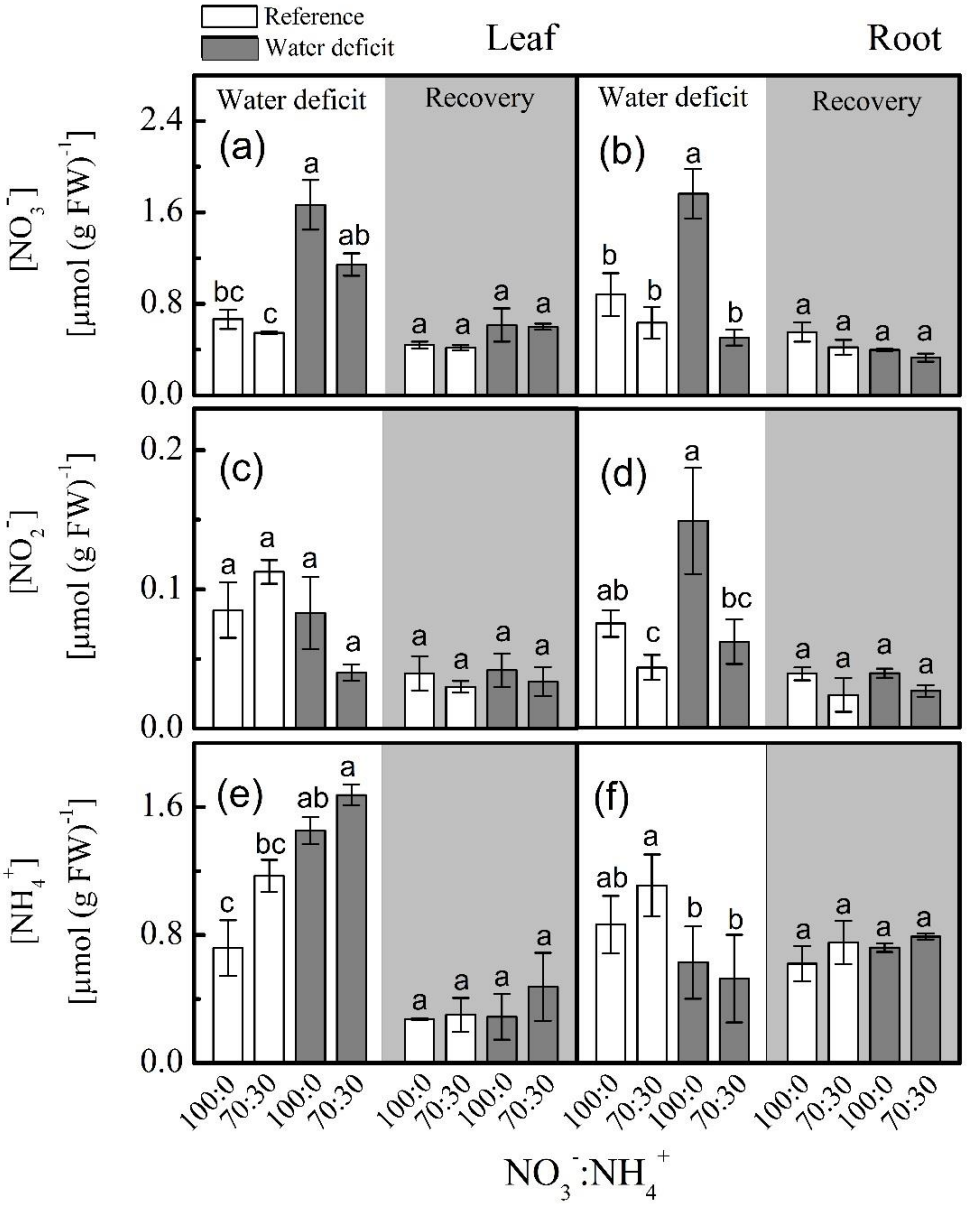
Reference
Water deficit
Leaf
Root
Water deficit
Recovery
Water deficit
Recovery
(a)
(b)
(c)
(d)
(e)
(f)
[NO3-] [µmol (g FW)-1]
[NO2-] [µmol (g FW)-1]
[NH4+] [µmol (g FW)-1]
2.4
1.6
0.8
0.0
0.2
0.1
0.0
1.6
0.8
0.0
100:0
70:30
100:0
70:30
100:0
70:30
100:0
70:30
100:0
70:30
100:0
70:30
100:0
70:30
100:0
70:30
NO3-:NH4+

Fig. 4

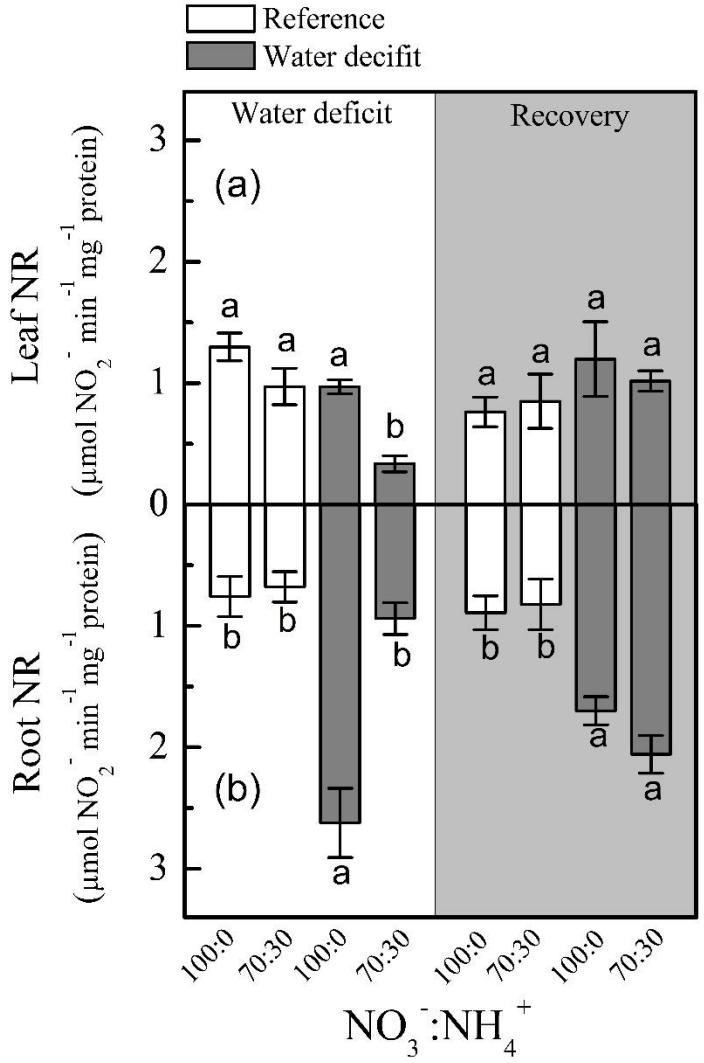
Reference
Water decifit
Water deficit
Recovery
(a)
(b)
Leaf NR
(μmol NO2- min-1 mg-1 protein)
Root NR
(μmol NO2- min-1 mg-1 protein)
3
2
1
0
1
2
3
a
a
a
b
b
b
b
a
a
a
b
b
b
a
a
a
100:0
70:30
100:0
70:30
100:0
70:30
100:0
70:30
NO3-:NH4+

Fig. 5

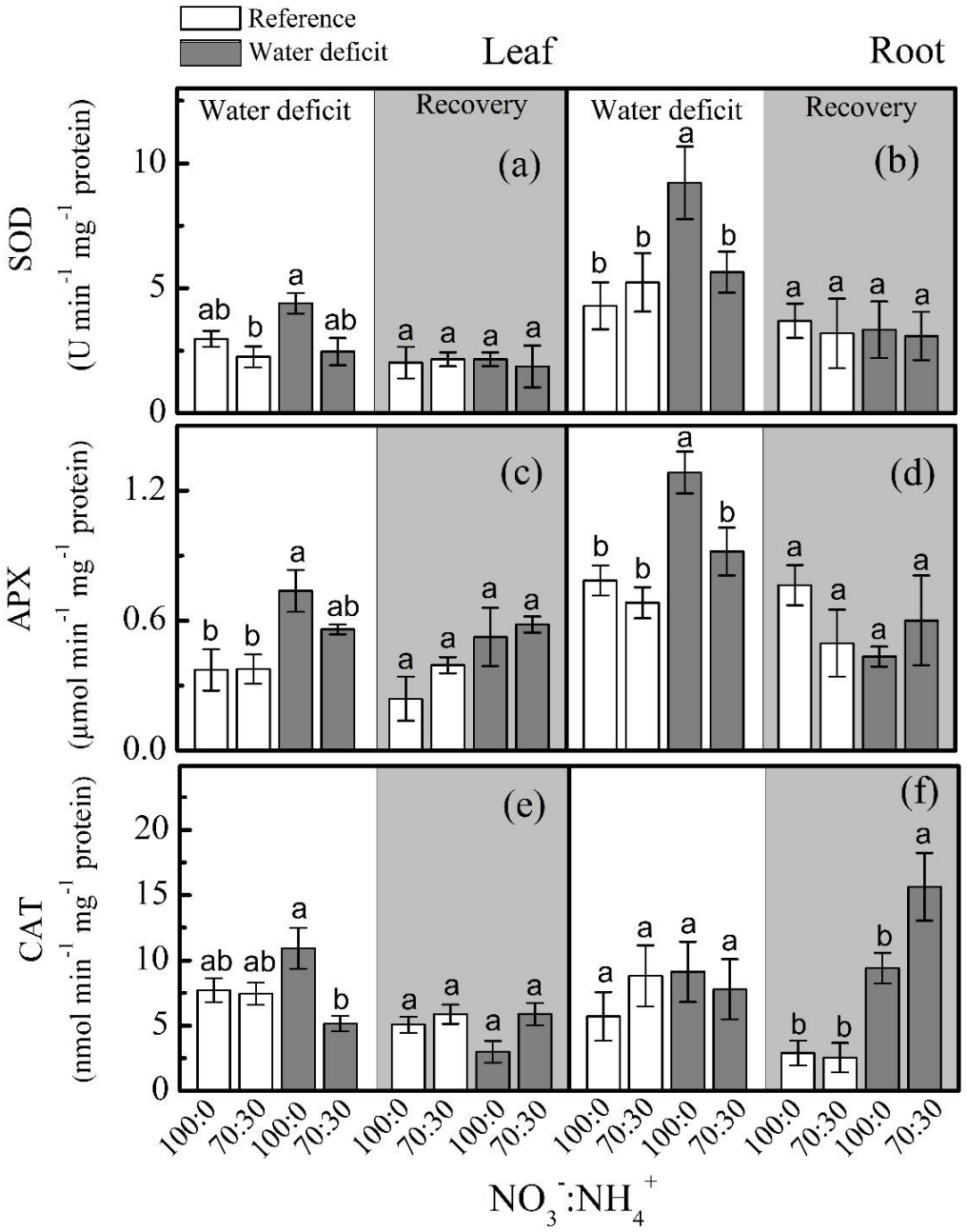
Reference
Water deficit
Leaf
Root
Water deficit
Recovery
Water deficit
Recovery
SOD
(U min-1 mg-1 protein)
APX
(µmol min-1 mg-1 protein)
CAT
(nmol min-1 mg-1 protein)
(a)
(b)
(c)
(d)
(e)
(f)
100:0
70:30
NO3-:NH4+

Fig. 6

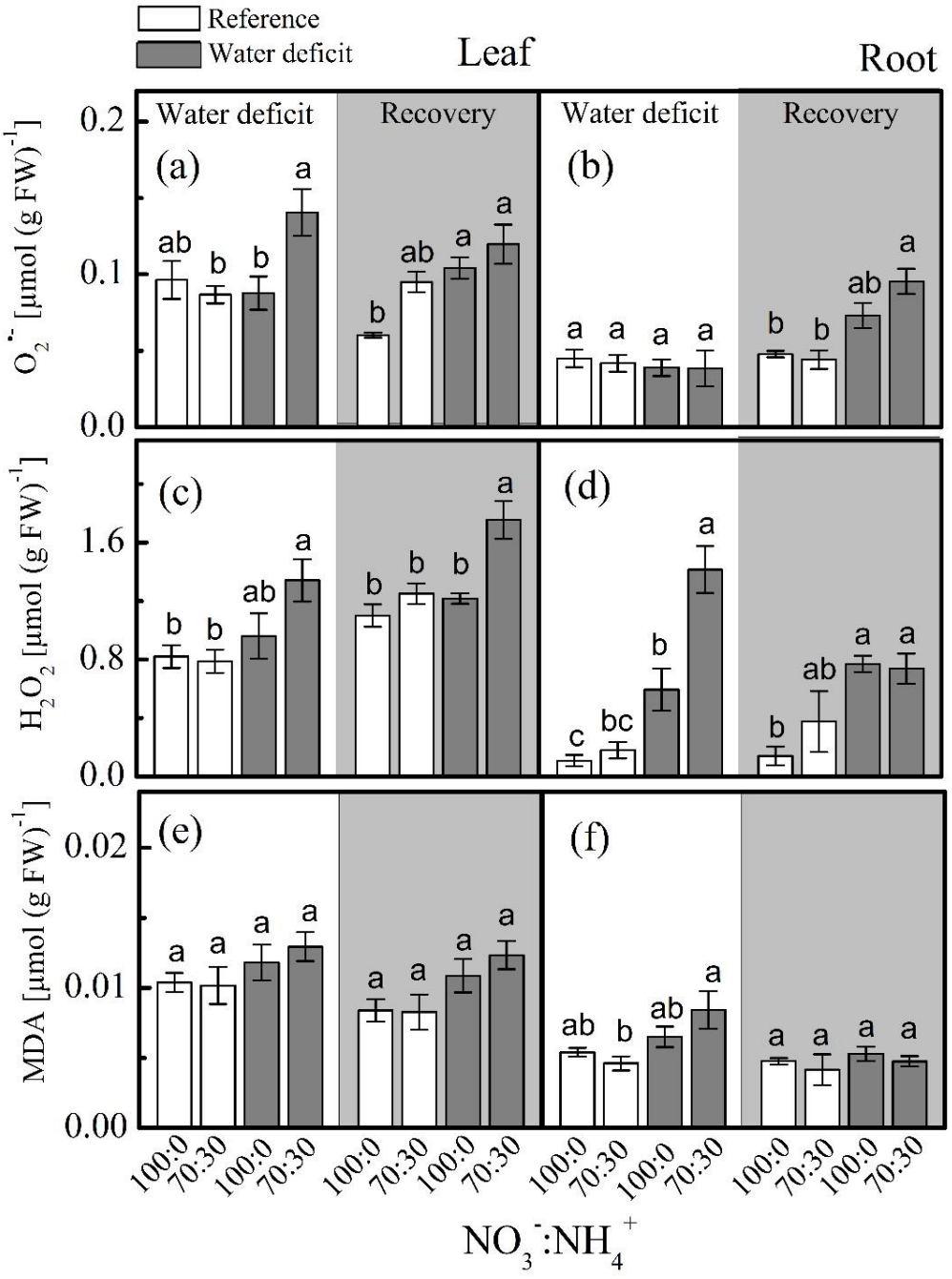
Reference
Water deficit
Leaf
Root
Water deficit
Recovery
Water deficit
Recovery
(a)
(b)
(c)
(d)
(e)
(f)
$O_2^{\bullet-}$ [µmol (g FW)$^{-1}$]
H_2O_2 [µmol (g FW)$^{-1}$]
MDA [µmol (g FW)$^{-1}$]
0.0
0.1
0.2
0.8
1.6
0.00
0.01
0.02
100:0
70:30
NO_3^-:NH_4^+

Fig. 7

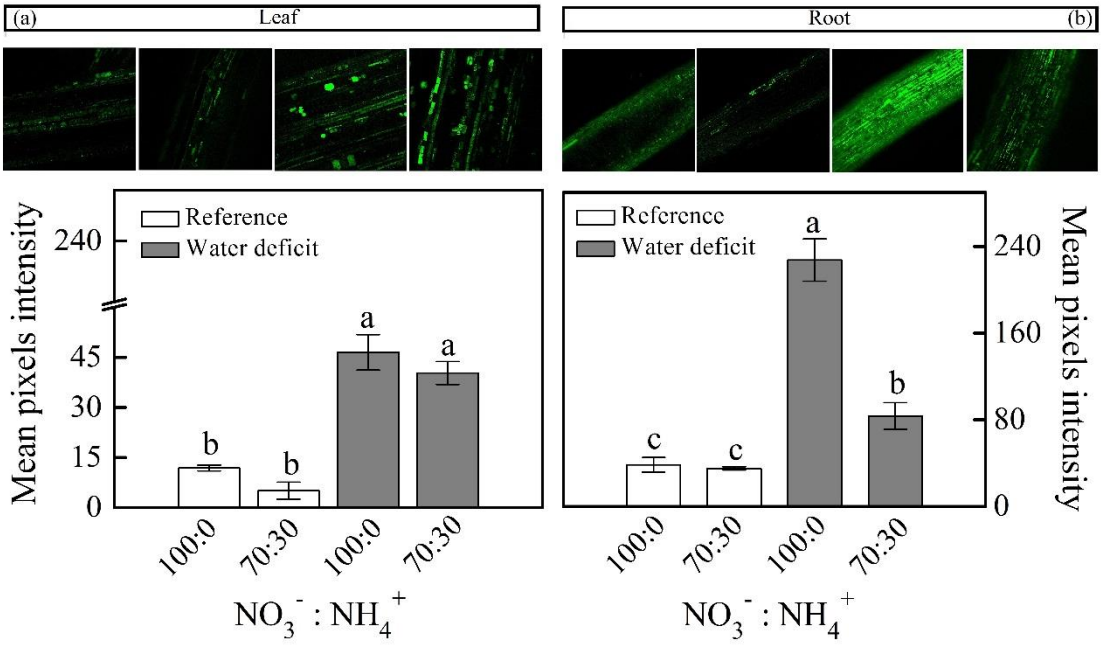
(a) Leaf
Root (b)
Reference
Water deficit
Mean pixels intensity
240
45
30
15
0
160
80
a
b
c
100:0
70:30
NO_3^- : NH_4^+

Fig. 8

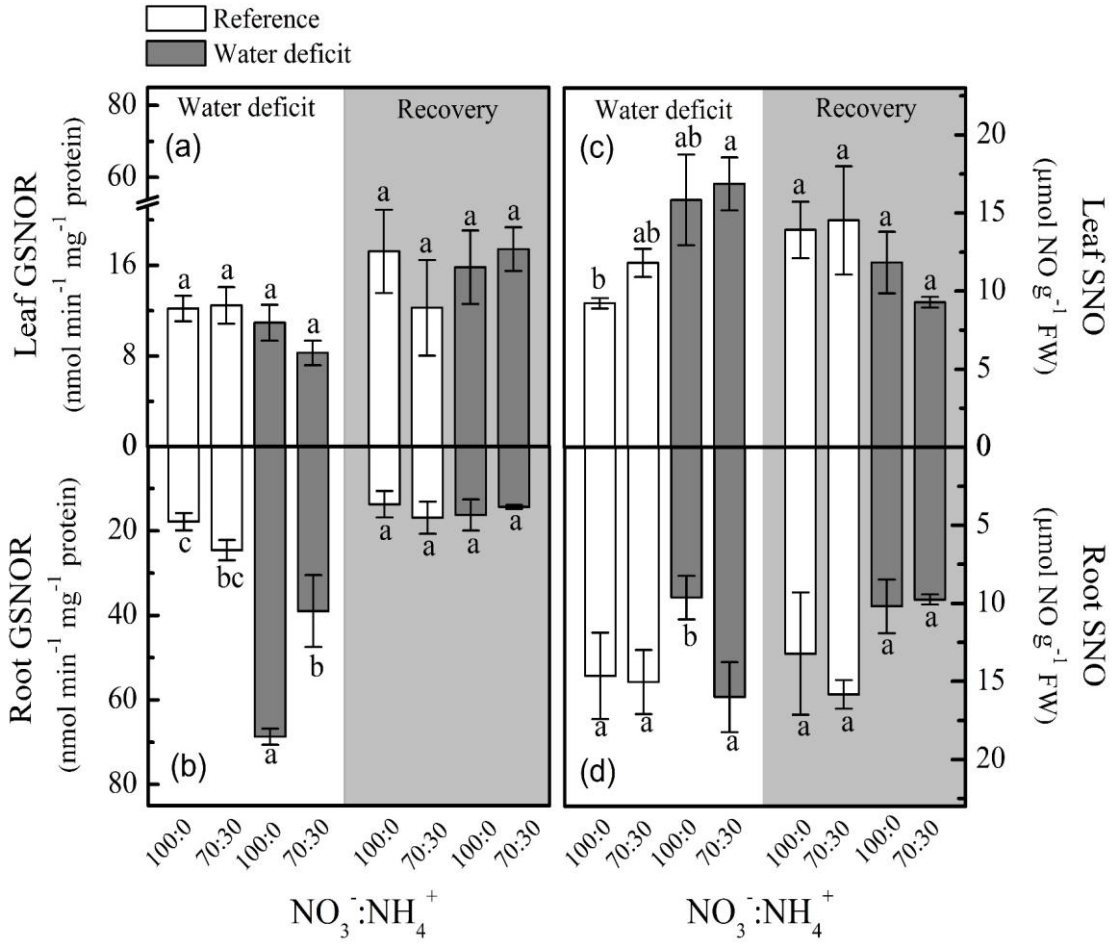
Reference
Water deficit
Water deficit
Recovery
(a)
(b)
(c)
(d)
Leaf GSNOR
(nmol min-1 mg-1 protein)
Root GSNOR
(nmol min-1 mg-1 protein)
Leaf SNO
(µmol NO g-1 FW)
Root SNO
(µmol NO g-1 FW)
100:0
70:30
100:0
70:30
100:0
70:30
100:0
70:30
NO3-:NH4+
NO3-:NH4+

Fig. 9

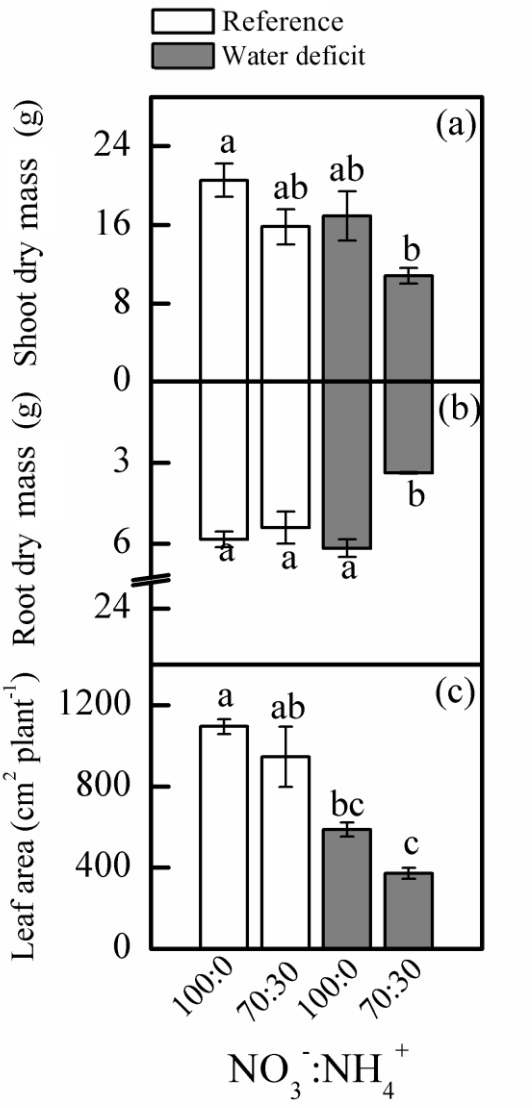
Reference
Water deficit
Shoot dry mass (g)
Root dry mass (g)
Leaf area (cm2 plant-1)
(a)
(b)
(c)
24
16
8
0
3
6
24
1200
800
400
0
a
ab
ab
b
a
a
a
b
a
ab
bc
c
100:0
70:30
100:0
70:30
NO3-:NH4+

Fig. 10

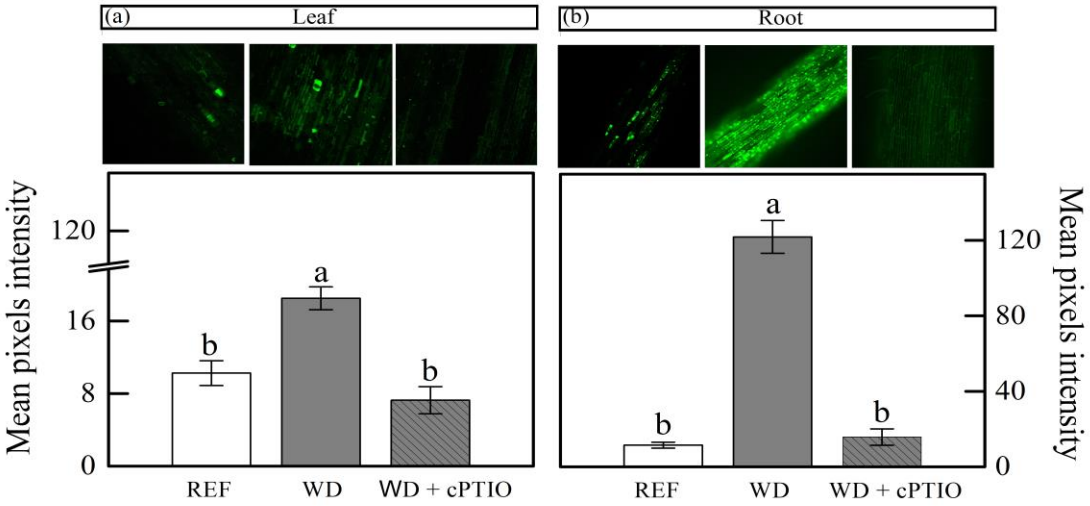
(a) Leaf
(b) Root
Mean pixels intensity
120
16
8
0
a
b
b
REF
WD
WD + cPTIO
120
80
40
0
a
b
b
REF
WD
WD + cPTIO
Mean pixels intensity

Fig. 11

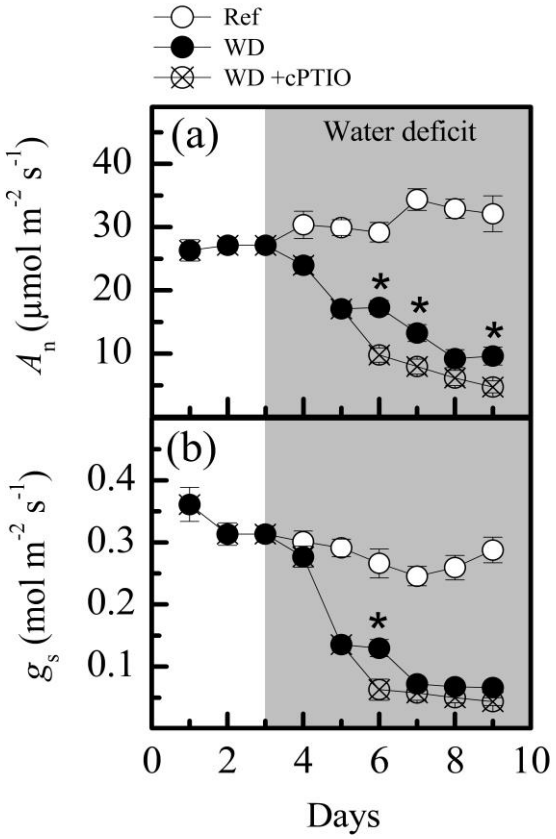
Ref
WD
WD +cPTIO
(a)
Water deficit
A_n (μmol m^{-2} s^{-1})
40
30
20
10
*
*
*
(b)
g_s (mol m^{-2} s^{-1})
0.4
0.3
0.2
0.1
*
0
2
4
6
8
10
Days

Fig. 12

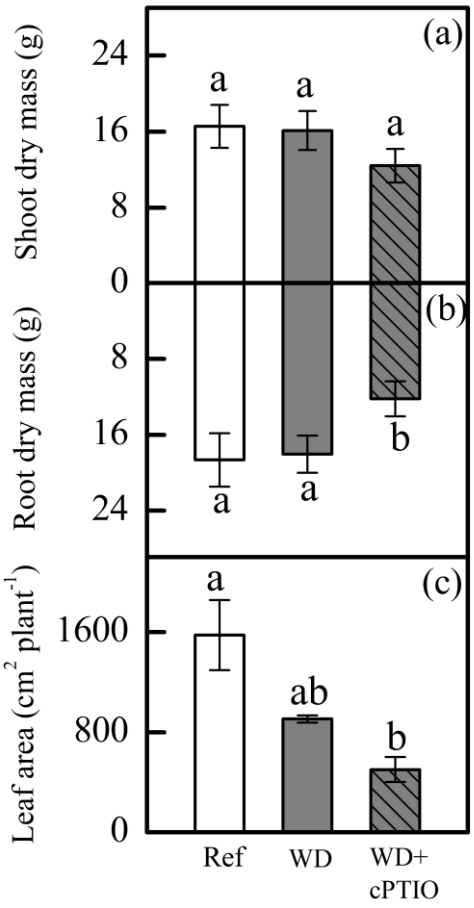
Shoot dry mass (g)
Root dry mass (g)
Leaf area (cm^2 plant^{-1})
(a)
(b)
(c)
24
16
8
0
8
16
24
1600
800
0
a
a
a
a
a
b
a
ab
b
Ref
WD
WD+
cPTIO

Fig. 13

Considerações finais

A cana-de-açúcar é uma cultura de grande relevância socioeconômica para o Brasil, e hoje é um pilar estratégico na matriz energética brasileira, produzindo etanol e energia elétrica, além de ser a principal fonte de açúcar. Em um cenário de mudanças climáticas e diminuição dos recursos hídricos, a escassez de água tornou-se um sério gargalo no rendimento das culturas em todo o mundo e os níveis de produção de cana-de-açúcar são extremamente dependentes das condições climáticas. Assim, o desenvolvimento de novas práticas e conceitos agrícolas sobre a tolerância à seca é de extrema importância para melhorar o rendimento das culturas.

Apesar dos diversos estudos que apontam o amônio como fonte preferencial de nitrogênio pela cana-de-açúcar, observamos que, quando mais de 30% desse íon está disponível no meio de cultura, estas plantas apresentam diversos efeitos negativos, sendo sensíveis ao amônio. Também foi possível concluir que pequenas alterações nas proporções de nitrato:amônio no momento da adubação podem ser um ponto chave para um melhor desempenho dessas plantas sob baixa disponibilidade hídrica, considerando o desenvolvimento inicial da cultura. O melhor desempenho observado na cana-de-açúcar que recebe mais nitrato como fonte de nitrogênio é devido ao aumento da produção de óxido nítrico (NO) nessas plantas, e está diretamente ligado ao aumento da atividade da nitrato redutase (NR), e apesar de controversas, consideramos aqui uma importante fonte enzimática da síntese deste radical. Assim, a sinalização redox mediada pelo NO desempenha um papel fundamental na defesa antioxidante, no crescimento e na melhoria da fotossíntese de plantas de cana-de-açúcar sob déficit hídrico.

Assim, a nutrição nitrogenada pode ser uma aliada das práticas agrícolas, beneficiando a cultura através de pequenas mudanças nas proporções de fonte de nitrogênio durante a fertilização. Como próximo passo, seria interessante realizar um estudo de campo para testar se os resultados obtidos no cultivo hidropônico seriam válidos no campo, e apesar dos diversos estudos sobre o nitrogênio, a interação entre sua oferta e a ocorrência de estresses ambientais ainda é pouco conhecido e requer mais estudos.

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Anexo 1

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Amenizando os efeitos do déficit hídrico em cana-de-açúcar com a nutrição nitrogenada**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 03 de setembro de 2019.

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Anexo 2

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "***Amenizando os efeitos do déficit hídrico em cana-de-açúcar com a nutrição nitrogenada***", desenvolvida no Programa de Pós-Graduação em Biologia Vegetal do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Data: 03 de setembro de 2019.