UNIVERSIDADE FEDERAL DE PELOTAS
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Tese

ADAPTAÇÕES METABÓLICAS DE GENÓTIPOS DE SOJA EM RESPOSTA À DEFICIÊNCIA DE OXIGÊNIO E ENVOLVIMENTO DO NITRATO

Junior Borella

Pelotas, 2015

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Tese apresentada ao Programa de Pós-graduação em Fisiologia Vegetal da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Fisiologia Vegetal.

Orientador: Dr. Luciano do Amarante

Coorientadores: Drª. Denise dos Santos Colares de Oliveira
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Tese aprovada, como requisito parcial, para obtenção do grau de Doutor em Fisiologia Vegetal, Programa de Pós-Graduação em Fisiologia Vegetal, Departamento de Botânica, Instituto de Biologia, Universidade Federal de Pelotas.

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Caitano e Elenir

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E a minha irmã,

Juliane

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"O que sabemos é uma gota, o que ignoramos é um oceano."

Isaac Newton

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Resumo

BORELLA, Junior. **Adaptações metabólicas de genótipos de soja em resposta à deficiência de oxigênio e envolvimento do nitrato.** 2015. 100f. Tese (Doutorado) – Programa de Pós-graduação em Fisiologia Vegetal, Instituto de Biologia, Universidade Federal de Pelotas, Pelotas, 2015.

O alagamento é um fator ambiental comum que causa deficiência de oxigênio às plantas, levando a uma inibição da respiração e redução do *status* energético celular, desencadeando uma série de mudanças no metabolismo do carbono e do nitrogênio. Além disso, alterações no fluxo de elétrons da cadeia de transporte de elétrons mitocondrial e cloroplastídica levam a produção de espécies reativas de oxigênio (EROs) que podem ocasionar vários danos ao metabolismo celular. No entanto, a aplicação exógena de nitrato tem sido reportada por promover efeitos benéficos em muitas espécies de plantas sob condições de hipóxia. Embora muitos estudos tenham sido envidados com soja, pouco se sabe a respeito das alterações metabólicas primárias do carbono e do nitrogênio que permitem diferenciar genótipos contrastantes ao alagamento e os efeitos no sistema antioxidante pela aplicação exógena de nitrato nas plantas. Considerando o exposto, os objetivos deste trabalho foram: I – avaliar mudanças no metabolismo do carbono e do nitrogênio e sua relação com a enzima alanina aminotransferase (AlaAT) em genótipos de soja nodulada; II – verificar possíveis efeitos benéficos no metabolismo antioxidante em plantas cultivadas na presença de nitrato (plantas não-noduladas) e na ausência de nitrato (plantas noduladas). Para isso, dois experimentos foram conduzidos em casa de vegetação com plantas de soja (*Glycine max* (L.) Merril) sob condições naturais de luz e temperatura. Experimento I: plantas noduladas de soja, nutridas na ausência de N mineral (Fundacep 53 RR – tolerante e BRS Macota – sensível) foram cultivadas em vermiculita e transferidas para sistema hidropônico, no estádio reprodutivo R2. O sistema radicular das plantas foi submetido à hipóxia pelo borbulhamento de nitrogênio gasoso na solução nutritiva diluída 1/3 da concentração normal, por 24 e 72 h. Para recuperação, após 72 h de hipóxia as plantas retornaram para vermiculita por 24 e 72 h. Foram avaliados, em raízes e nódulos, metabólitos fermentativos e ácidos orgânicos (GC-MS), aminoácidos (HPLC), expressão relativa dos genes (RT-PCR) e atividade da enzima AlaAT. Fundacep 53

RR acumulou mais teores de piruvato e lactato que BRS Macota e embora a composição de aminoácidos não tenha diferido entre os genótipos, foi observado uma ligação entre a glicólise e o ciclo dos ácidos tricarboxílicos via indução dos genes e atividade da AlaAT, que, posteriormente, levou ao acúmulo de succinato em raízes de Fundacep 53 RR, podendo aumentar o ganho energético em relação à BRS Macota sob hipóxia. Experimento II: A condução experimental adotada foi semelhante ao experimento I, no entanto conduzido com plantas noduladas e não-noduladas (nutridas com nitrato) de soja, de ambos os genótipos. Foi avaliado o sistema antioxidante em raízes e folhas através da atividade das enzimas superóxido dismutase (SOD), ascorbato peroxidase (APX), catalase (CAT), glutationa redutase (GR), guaiacol peroxidase (GPOD) e glutationa S-transferase (GST), o conteúdo de ascorbato reduzido e ascorbato total, bem como conteúdo de superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2) e peroxidação de lipídeos. O sistema antioxidante foi fortemente induzido nas raízes das plantas nutridas com nitrato de ambos os genótipos, com elevada atividade de SOD, APX, CAT, GR e GPOD, bem como o aumento do conteúdo de ascorbado reduzido e total e diminuição da produção de EROs em condições de hipóxia e de recuperação, enquanto que nas folhas de plantas noduladas e não-noduladas foi observado um ligeiro aumento nos componentes enzimáticos e não enzimáticos antioxidantes. O nitrato exerce efeitos benéficos em plantas de soja em condições de hipóxia e consequentemente na recuperação por induzir o sistema antioxidante nas raízes, permitindo modular os possíveis danos oxidativos causados pela produção de EROs, além de poder prolongar a tolerância dessas plantas.

Palavras-chave: *Glycine max*, alagamento, hipóxia, metabolismo do carbono, aminoácidos, sistema antioxidante.

Abstract

BORELLA, Junior. **Metabolic adaptations of soybean genotypes in response to low oxygen and involvement of nitrate.** 2015. 100p. Thesis (Ph.D.) – Post-Graduate Program in Plant Physiology, Institute of Biology, Federal University of Pelotas, Pelotas, 2015.

Waterlogging is a common environmental stress which causes oxygen deprivation in plants leading to an inhibition of the mitochondrial respiration. It leads to a reduction of cellular energy status triggering changes at different levels of carbon and nitrogen metabolism. In addition, it leads to electron scape from the mitochondrial and chloroplast electron transport chain, producing reactive oxygen species (ROS) which cause severe oxidative damage to cells. However, exogenous nitrate supply has been reported to promoting beneficial effects in several plant species under hypoxic conditions. Although many studies have been carried out with soybean, a little is known about the primary metabolic changes in carbon and nitrogen metabolism, which may differ between tolerant and sensitive plant genotypes in response to waterlogging and the effects on antioxidant system in nitrate-supplied plants in comparison to non-nitrate-supplied plants. Thus, the aims of this study were: I – to evaluate the hypoxia-induced alterations of carbon and nitrogen metabolism and its relation with alanine aminotransferase (AlaAT) enzyme in nodulated soybean genotypes; II – to verify possible beneficial effects on antioxidant metabolism in nitrate-supplied plants (non-nodulated plants) in comparison to plants growing in absence of nitrate (nodulated plants). For that, two experiments were carried out in greenhouse under natural light and temperature conditions. Experiment I: Nodulated soybean plants (Fundacep 53 RR – tolerant and BRS Macota – sensitive) were grown in vermiculite and transferred to hydroponic system at reproductive stage. Root system was subjected to hypoxia by flushing N_2 gas into the solution for 24 or 72 h. For the recovery, after 72 h in hypoxia, plants returned to normoxic conditions by transferring back to vermiculite for 24 and 72 h. Root and nodule organic acids and amino acids were analysed by gas chromatography-mass spectrometry and high-performance liquid chromatography, respectively. Relative expression of *AlaAT* (qRT-PCR) and AlaAT activity were also verified in both genotypes. Plants of Fundacep 53 RR and BRS Macota genotypes responded distinctly upon hypoxia. Fundacep 53 RR presented higher pyruvate and lactate accumulation than BRS Macota, which is indicative of higher glycolytic and fermentation rates in root tissues.

Furthermore, Fundacep 53 RR responds more effectively to the recovery by restoring pre-hypoxic levels of the metabolites. Although the amino acid composition did not differ between the genotypes, there was a clear link between glycolysis and the TCA via increase of gene expression and activity of AlaAT enzyme by leading a succinate accumulation in Fundacep 53 RR, wich represents a metabolic advantage compared to BRS Macota under hypoxic stress. Experiment II: was carried out in a similar way of Experiment I, however with plants growing in presence (non-nodulated) and absence (nodulated) of nitrate, for both soybean genotypes. Superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), guayacol peroxidase (GPOD) and glutathione S-transferase (GST) enzymes; reduced ascorbate and ascorbate redox state; superoxide content ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and lipid peroxidation were analysed in roots and leaves of both soybean genotypes. Antioxidative system was strongly induced in roots of nitrate-supplied plants of both genotypes, with high activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and guayacol peroxidase (GPOD), as well as increased ascorbate reduced and ascorbate redox state and decreased ROS production under hypoxia and recovery, while in leaves of nodulated and non-nodulated plants a slight increase on antioxidant system was observed. Furthermore, the results did not show tolerance differences between the genotypes. Nitrate exerts beneficial effects in soybean plants under hypoxic conditions and consequent recovery by inducing the antioxidant system manly in roots, to cope possible oxidative damage caused by ROS production and also can postpone the effects of hypoxia in both genotypes.

Key words: *Glycine max*, waterlogging, hypoxia, carbon metabolism, amino acids, antioxidant system.

Lista de Figuras

Lista de abreviaturas

$^1O_2$	oxigênio singleto
ADH	álcool desidrogenase
Ala	alanina
AlaAT	alanina aminotransferase
ANOVA	análise de variância
AOX	oxidase alternativa
APX	ascorbato peroxidase
AsA	ascorbato
Asn	asparagina
Asp	aspartato
AspAT	aspartato aminotransferase
ATP	adenosina trifosfato
Ca^{2+}	cálcio
CAT	catalase
Cb_6f	citocromo
CDNB	2,4-dinitroclorobenzeno
CoA	coenzima A
COX	citocromo *c* oxidase
CytC	citocromo c
DHA	dehidroascorbato
DHAR	dehidroascorbato redutase
DNA	ácido desoxirribonucleico
DTT	ditiotreitol
EDTA	ácido etilenodiamino tetra-acético
EM	enzima málica
EROs/ROS	espécies reativas de oxigênio

Fd	ferredoxina
$FeCl_3$	cloreto férrico
FFAs	ácidos graxos livres
FSI	fotossistema I
FSII	fotossistema II
GABA	ácido *γ*–aminobutírico
GABA-T	ácido γ-aminobutírico transaminase
GAD	glutamato descarboxilase
GC-MS	cromatografia gasosa-espectrometria de massas
Gln	glutamina
Glu	glutamato
GOGAT	glutamina-oxoglutarato aminotransferase
GPOD	guaiacol peroxidase
GPX	glutationa peroxidase
GR	glutationa redutase
GSH	glutationa reduzida
GSSG	glutationa oxidada
GST	glutationa S-transferase
H_2O_2	peróxido de hidrogênio
H_3PO_4	ácido fosfórico
HCl	ácido clorídrico
HPLC	cromatografia líquida de alta eficiência
I, II, III e IV	complexos da cadeia de transporte de elétrons mitocondrial
INV	invertase
LDH	lactato desidrogenase
MCW	metanol, clorofórmio e água
MDA	monodehidroascorbato
MDAR	monodehidroascorbato redutase
MDH	malato desidrogenase
N	nitrogênio
NAD^+	nicotinamida adenina dinucleotídeo
NADH	nicotinamida adenina dinucleotídeo reduzido
$NADP^+$	nicotinamida adenina dinucleotídeo fosfato

NADPH	nicotinamida adenina dinucleotídeo fosfato reduzido
NBT	azul de nitro-tetrazólio
ND	desidrogenase interna e externa
NH_3	amônia
NH_4^+	amônio
NO	óxido nítrico
NO_2^-	nitrito
NO_3^-	nitrato
NR	nitrato redutase
nsHb	hemoglobina não simbionte
NT	transportador de nitrito
O_2	oxigênio
$O_2^{\bullet-}$	superóxido
OAA	oxaloacetato
OGDH	2-oxoglutarato desidrogenase
$OH^{\bullet}$	radical hidroxila
OPA	*o*-fitaldialdeído
PC	plastocianina
PDC	piruvato descarboxilase
PEP	fosfoenolpiruvato
PEPC	fosfoenolpiruvato carboxilase
PPi	pirofosfato
PVPP	polivinilpolipirrolidona
RNA	ácido ribonucleico
RT-PCR	reação em cadeia da polimerase em tempo real
SAS	statistical analysis system
SCS	succinil-CoA ligase
SDH	succinato desidrogenase
Ser	serina
SOD	superóxido dismutase
SSA	semialdeído succínico
SSADH	semialdeído succínico desidrogenase
SUS	sacarose sintase

TBA	ácido tiobarbitúrico
TCA	ácido tricloro ácetico
TCA	ciclo dos ácidos tricarboxílicos
UCP	proteína desacopladora
UQ	ubiquinona
V	ATP-sintase

Sumário

Introdução Geral

Os eventos de alagamento ou de inundações do solo têm se tornado cada vez mais frequentes devido às fortes chuvas em consequência das mudanças climáticas, gerando dificuldades para a produção agrícola e florestal em diversas regiões do mundo (JACKSON; COLMER, 2005; KUMUTHA et al., 2008; BAILEY-SERRES; COLMER, 2014; LIMAMI et al., 2014). A América do Sul possui vários ambientes sujeitos a inundações, como florestas de galeria, o Pantanal e os chamados solos de várzea resultantes de um alagamento temporário na época das chuvas ou em cheias de rios (ANDRADE et al., 1999). No Brasil, aproximadamente 28 milhões de hectares de solos estão sujeitos ao encharcamento (solos aluviais e hidromórficos), parte encontra-se na região dos Cerrados e parte na região Sul do Brasil (VITORINO et al., 2001; MAGALHÃES et al., 2005). Cerca de 5,4 milhões de hectares situam-se no Estado do Rio Grande do Sul e poderiam ser incorporados ao processo produtivo (SCOTT; NORMAN, 2000).

A América do Sul situa-se em uma região susceptível a altas influências devido às mudanças climáticas (IPCC, 2014), entre elas, um aumento considerável no número de inundações registrado nas últimas seis décadas (BAILEY-SERRES et al., 2012). Em oposição às perdas em produtividades, principalmente agrícolas, que esses eventos ocasionam está a estimativa de aumento populacional global para os próximos anos e a crescente demanda por alimentos (FAO, 2009).

O alagamento tem um efeito drástico levando a redução do oxigênio no solo e restringindo a respiração aeróbica nas raízes (BAILEY-SERRES; VOESENEK, 2008; BAILEY-SERRES et al., 2012). A maioria das plantas são sensíveis a condições de hipóxia no sistema radicular e principalmente em completa submergência, a exceção do arroz que apresenta uma excepcional tolerância ao alagamento (BAILEY-SERRES; COLMER, 2014). No entanto, as plantas têm mostrado uma grande variação em sua habilidade de lidar com as limitações de oxigênio no solo, através de uma série de mecanismos adaptativos para garantir sua sobrevivência ou remediar os efeitos ocasionados pela hipóxia (SOUZA; SODEK, 2002; MOMMER et

al., 2005; THOMAS et al., 2005; BAILEY-SERRES; VOESENEK, 2008; BAILEY-SERRES et al., 2012).

Desse modo, uma forma de aumentar a eficiência do sistema produtivo é diversificar as espécies cultivadas nas áreas de várzea (sujeitas ao alagamento), através do melhoramento e incorporação de genótipos mais tolerantes, como a soja, em rotação de cultura com o arroz irrigado (SCOTT; NORMAN, 2000), uma vez que o Brasil encontra-se em uma das regiões com maior potencial de expansão em produtividade agrícola (FAO, 2009). Além disso, a rotação de culturas ajuda a preservar ou melhorar as características físicas, químicas e biológicas do solo; o controle de plantas daninhas, doenças e pragas; além de contribuir com a incorporação de N no solo devido a associação com rizóbios responsáveis pela fixação do nitrogênio atmosférico em plantas leguminosas, representando economia em fertilizantes nitrogenados (EMBRAPA SOJA, 2013).

O Brasil é um dos maiores produtores de soja do mundo, com produção estimada em 88 milhões de toneladas em 2014 (IBGE, 2015). Além da importância econômica mundial, a soja é uma leguminosa que oferece proteínas de alta qualidade (BALESTRASSE et al., 2001). Nas condições brasileiras, a cultura de soja, principal produtora de óleo vegetal, matéria-prima para a produção de biodiesel, apresenta-se como uma alternativa interessante e potencialmente viável para ocupar esse segmento (EMBRAPA SOJA, 2013).

A soja também apresenta uma cadeia produtiva bem estruturada, com uma ampla rede de pesquisa que assegura soluções rápidas para possíveis problemas associados à cultura, oferece rápido retorno do investimento e é de fácil comercialização. Embora seja uma cultura amplamente difundida, o seu cultivo em solos sujeitos ao alagamento de forma a possibilitar retorno econômico, depende da existência de cultivares tolerantes ao excesso de água no solo. Por ser espécie originária de áreas alagadiças do norte da China (EVANS, 1996), apresenta variabilidade genética para tolerar o excesso de umidade no solo que precisa ser explorado (THOMAS et al., 2000).

Os vegetais superiores necessitam de grande demanda de O_2 para manter o metabolismo e o crescimento. Em condições de campo, é comum ocorrer deficiência de oxigênio no sistema radicular das plantas (KENNEDY et al., 1992) especialmente das plantas cultivadas durante o seu ciclo (JACKSON et al., 1982), pois mesmo solos bem drenados tornam-se encharcados por curtos períodos, depois de fortes

chuvas, submetendo as raízes a ambientes hipóxicos (ARMSTRONG et al., 1994; FUKAO; BAILEY-SERRES, 2004; BAILEY-SERRES; VOESENEK, 2008; PEDERSEN et al., 2009; BAILEY-SERRES; COLMER, 2014; LIMAMI et al., 2014).

A limitação de oxigênio afeta negativamente o desempenho da planta (GEIGENBERGER, 2003; BAILEY-SERRES; VOESENEK, 2008; HORCHANI et al., 2009), principalmente das raízes que requerem fornecimento suficiente para atingirem as suas funções plenamente (van DONGEN et al., 2003; ARMSTRONG et al., 2009; ZABALZA et al., 2009). Em solos alagados, o suprimento de O_2 aos órgãos submersos é insuficiente devido à baixa difusão dos gases na água, 10.000 vezes menor em relação ao ar (ARMSTRONG et al., 1994), levando ao desencadeamento do estresse hipóxico nas plantas. Assim, a sobrevivência das espécies vegetais ou desenvolvimento de tolerância à deficiência de O_2 depende de uma série de mecanismos adaptativos que ocorrem em três estágios. Inicialmente, a planta induz rapidamente uma série de componentes de transdução de sinal. Esse evento é seguido por adaptações metabólicas, envolvendo as rotas primárias do carbono e nitrogênio e, finalmente, dependendo da tolerância da espécie, há o desenvolvimento de mudanças morfológicas como aerênquima e/ou formação de raízes adventícias (DENNIS et al., 2000; BAILEY-SERRES; VOESENEK, 2008; BAILEY-SERRES et al., 2012; KREUZWIESER; RENNENBERG, 2014; SHINGAKY-WELLS et al., 2014).

Metabolismo de carboidratos e do nitrogênio sob deficiência de oxigênio

As plantas respondem ao estresse por déficit de O_2 ativando a via de metabolismo anaeróbico (SACHS et al., 1980), desencadeando várias mudanças metabólicas, dentre as quais, a obtenção de energia passa a ser principalmente pela via glicolítica em detrimento à fosforilação oxidativa mitocondrial (TADEGE et al., 1999; KUMUTHA et al., 2008; HORCHANI et al., 2009; ZABALZA et al., 2009). Sob essas condições, o “efeito Pasteur” é desencadeado levando ao catabolismo de carboidratos a fim de manter, embora baixa, a produção de ATP através da glicólise (MAGNESCHI; PERATA, 2009). A glicólise produz dois ATP e duas moléculas de piruvato por unidade de hexose (SOUSA; SODEK, 2002; SAÍRAM et al., 2009), enquanto concomitantemente, ocorre a redução do NAD^+ para NADH. A fim de manter a glicólise sob condições anóxicas, o NAD^+ precisa ser continuamente

regenerado a partir de NADH via reações fermentativas (Fig. 1) (ARMSTRONG et al., 1994; RICARD et al., 1994; DREW, 1997; TADEGE et al., 1999; SOUSA; SODEK, 2002; ZABALZA et al., 2009; BAILEY-SERRES et al., 2012; van DONGEN; LICAUSI, 2015).

Numa tentativa de reduzir a demanda por energia celular, a degradação da sacarose via glicólise em muitas espécies de plantas ocorre pela enzima sacarose sintase (SUS) e outras que utilizam PPi (pirofosfato) em vez de ATP, ao invés da invertase (INV) que é inibida sob tais condições (KUMUTHA et al., 2008; SAIRAM et al., 2009; MUSTROPH et al., 2014). A SUS foi descoberta como sendo um dos maiores polipeptídios anaeróbicos transcritos em milho (SPRINGER et al., 1986), soja (NANJO et al., 2011) e outras espécies (HARADA et al., 2005; CHRISTIANSON et al., 2010; MUSTROPH et al., 2010). Embora, recentemente, tenha sido proposto que a SUS em *Arabidopsis* não é via preferencial do catabolismo da sacarose, uma vez que mutantes para SUS exibiram alta conversão de sacarose em glicose e frutose (SANTANIELLO et al., 2014).

Em soja, o alagamento do sistema radicular tem ocasionado o amarelecimento e abscisão das folhas dos nós inferiores, diminuição no crescimento, na fotossíntese, no rendimento de grãos (SCOTT et al., 1989; THOMAS et al., 2000), na absorção de nutrientes e na fixação e assimilação do nitrogênio (PUIATTI; SODEK, 1999; SOUZA; SODEK, 2002; AMARANTE; SODEK, 2006). O suprimento de carboidratos e a regulação do metabolismo de carboidratos e de energia são importantes na superação do estresse hipóxico (ANDREEV et al., 1991; KUMUTHA et al., 2008) levando muitas plantas ao acúmulo de aminoácidos (FAN et al., 1988; ROCHA et al., 2010a; SHINGAKI-WELLS et al., 2011) e carboidratos, como sacarose (SAÍRAM et al., 2009) e amido (BARTA, 1987) quando submetidas à deficiência de O_2.

O aumento dos teores de carboidratos, nas raízes e principalmente na parte aérea, ocorre quando as raízes são submetidas ao estresse hipóxico, mesmo havendo diminuição na taxa fotossintética (SAIRAM et al., 2009). Algumas pesquisas sugerem que o acúmulo desses compostos se deve pela diminuição na taxa de crescimento (BARRET-LENNARD et al., 1988) e pela diminuição da taxa de respiração (HUANG; JOHNSON, 1995).

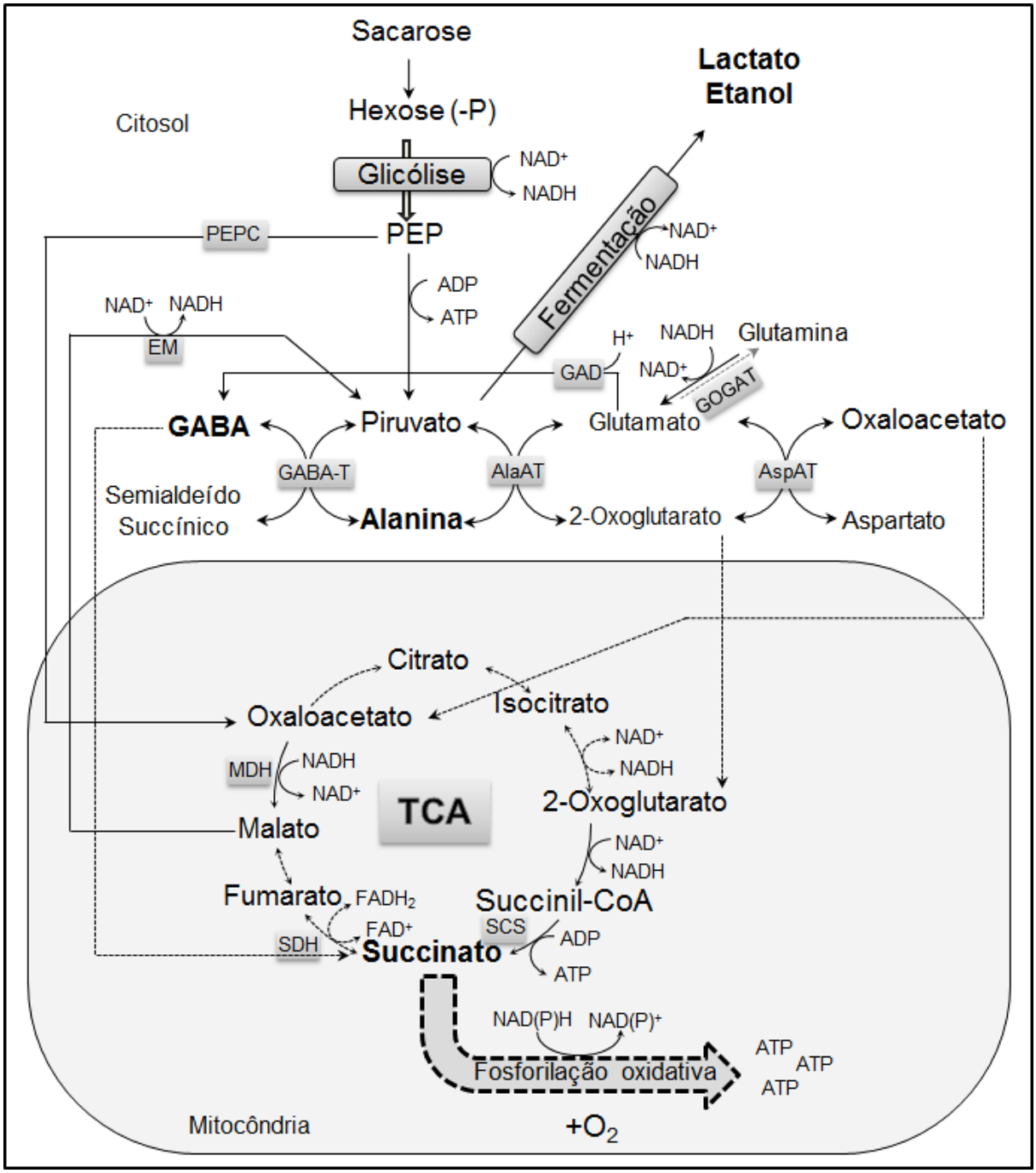

Figura 1 - Esquema representativo da utilização de carboidratos e intermediários metabólicos-chave em plantas submetidas à hipóxia do sistema radicular (vias principais e alternativas) [Adaptado de Rocha et al. (2010a)]. Abreviações: PEP – fosfoenolpiruvato; PEPC – fosfoenolpiruvato carboxilase; GAD - glutamato descarboxilase; GOGAT - glutamina-oxoglutarato aminotransferase; GABA-T - ácido γ-aminobutírico transaminase; AlaAT - alanina aminotransferase; AspAT - aspartato aminotransferase; EM - enzima málica; MDH - malato desidrogenase; SCS - succinil-CoA ligase; SDH - succinato desidrogenase; TCA - ciclo dos ácidos tricarboxílicos.

A ativação do metabolismo fermentativo desencadeia um acúmulo de produtos, como o etanol, lactato e alanina (Ala) principalmente (SOUZA; SODEK, 2002; ROCHA et al., 2010a), derivados do piruvato, produto final da glicólise (DREW, 1997). Embora a indução da atividade das enzimas fermentativas possa contribuir para a sobrevivência e superar a escassez de energia através da fermentação de carboidratos para manter a produção de ATP na ausência de oxigênio (WANG et al., 2009), o benefício sob tais condições vai depender, também, do tipo de tecido, estádio de desenvolvimento, espécie, genótipo, da gravidade e da duração do estresse (FUKAO; BAILEY-SERRES, 2004; WANG et al., 2009). De modo geral, espécies ou genótipos que apresentam maior concentração de carboidratos nas raízes e um mecanismo metabólico eficiente associado a sua mobilização via metabolismo fermentativo, apresentam maior tolerância para enfrentar a privação de oxigênio (SAIRAM et al., 2009).

Usando o piruvato como substrato, o metabolismo fermentativo produz lactato através da lactato desidrogenase (LDH) ou etanol através de duas reações subsequentes catalisada pela piruvato descarboxilase (PDC) e álcool desidrogenase (ADH) (TADEGE et al., 1999; ZABALZA et al., 2009; YANG et al., 2014). No entanto, estas duas vias têm desvantagens claras: lactato é tóxico para as células e etanol se difunde rapidamente para fora das células, o que leva a uma perda considerável de carbono durante a hipóxia (ROCHA et al., 2010a).

Além de lactato e etanol, muitas espécies vegetais acumulam alanina (Ala) e GABA em condições hipóxicas (SOUZA; SODEK, 2003; MIYASHITA et al., 2007). A Ala é um dos aminoácidos fortemente acumulado em resposta à anaerobiose e o aumento se deve à interconversão entre aminoácidos como glutamato, glutamina e aspartato (SOUZA; SODEK, 2003; ROCHA et al., 2010a;b).

As plantas adquirem o nitrogênio a partir da solução do solo, na forma de íons inorgânicos como o amônio (NH_4^+) e o nitrato (NO_3^-) ou a partir do nitrogênio atmosférico via associação simbiótica. Assim, a assimilação em aminoácidos e o transporte dessa molécula são profundamente alterados durante o estresse hipóxico (SHINGAKI-WELLS et al., 2011; JUSTINO; SODEK, 2013; OLIVEIRA et al., 2013a). Ao ser absorvido pelas raízes, o NO_3^- é reduzido a nitrito (NO_2^-) pela nitrato redutase (NR) e, por sua vez, reduzido a amônio pela nitrito redutase (NiR). O amônio é então incorporado em aminoácidos pelo sistema glutamina sintetase-glutamina-2-oxoglutarato aminotransferase (GS-GOGAT) (UDVARDI; POOLE, 2013).

A redução e assimilação do nitrato pode ocorrer mesmo sob condições de hipóxia (REGGIANI et al., 1995; OLIVEIRA et al., 2013a,b). Os resultados sobre os efeitos da deficiência de O_2 no aumento ou decréscimo da atividade da NR são controversos, demonstrando forte inibição da NiR nessas condições (BOTREL et al., 1996, BRANDÃO; SODEK, 2009). Em estudo com fontes de nitrogênio marcado (^{15}N) foi observada uma limitação do metabolismo do nitrato em segmentos de raízes de milho (LIBOUREL et al., 2006), enquanto em outros demonstrado a incorporação de ^{15}N em aminoácidos, detectados em coleóptilos de arroz sob condições anaeróbicas de germinação (REGGIANI et al., 1995). A assimilação do amônio é também afetada devido à inibição da reação catalisada pela GS, provavelmente pela necessidade de ATP. Baixa incorporação de amônio marcado ($^{15}NH_4^+$) foi observado sob condições de hipóxia em plântulas de *Medicago truncatula* (LIMAMI et al., 2008).

A hipóxia induz muitas alterações no metabolismo de aminoácidos (REGGIANI; BERTANI, 2003) e, independente, do sistema de assimilação do nitrogênio (condição simbiótica ou não simbiótica), provoca alterações marcantes na composição de compostos nitrogenados transportados no xilema (PUIATTI; SODEK, 1999; OLIVEIRA et al., 2013a).

A produção de Ala é importante, pois confere tolerância às plantas sob deficiência de O_2, é um produto do metabolismo que não causa toxidez à célula (DREW, 1997) e fornece um “pool” de reserva de nitrogênio que poderia ser usado para a síntese de aminoácidos, após o retorno à normóxia, pela sua conversão em piruvato por meio da reação de transaminação catalisada pela atividade da enzima alanina aminotransferase (AlaAT) (SOUZA; SODEK, 2003; MIYASHITA et al., 2007; MIYASHITA; GOOD, 2008). Essa enzima catalisa a reação reversível da interconversão de piruvato e glutamato em alanina e 2-oxoglutarato, atuando tanto no metabolismo do carbono quanto no metabolismo de nitrogênio em plantas (MIYASHITA et al., 2007).

É ainda sugerido que a produção de Ala ajuda a regular o equilíbrio do pH dentro de uma célula anóxica (REGGIANI et al., 1988). No entanto, ainda não está claro como exatamente o papel fisiológico da produção de Ala ajudaria o metabolismo anóxico, visto que não ocorre a oxidação de NADH durante a sua produção (SOUZA; SODEK, 2002).

Várias vias metabólicas têm sido propostas para explicar o acúmulo de Ala sob condições de hipóxia/anóxia. A rápida indução da expressão do gene que codifica a enzima AlaAT, bem como um aumento na atividade da enzima durante inundações têm sido bem documentados (GOOD; CROSBY, 1989; GOOD; MUENCH, 1993; SOUZA; SODEK, 2003; MIYASHITA et al., 2007; ROCHA et al., 2010b). As transaminases estão entre as enzimas mais importantes em plantas sob metabolismo anaeróbico, no entanto são enzimas poucos estudadas, com exceção de alguns trabalhos (GOOD; CROSBY, 1989; ROCHA et al., 2010a;b).

Rocha et al. (2010a) descreveram a ligação entre a glicólise e o ciclo do ácido tricarboxílico (TCA) com a atividade da alanina aminotransferase durante a hipóxia induzida pelo alagamento de *Lotus japonicus*, propondo um modelo metabólico do papel da AlaAT. Um resumo desse modelo pode ser descrito através da reação do piruvato com o glutamato para formar Ala e 2-oxoglutarato via AlaAT. Isso evita o acúmulo de piruvato e, simultaneamente, 2-oxoglutarato é produzido e pode, nas mitocôndrias, formar succinato via 2-oxoglutarato desidrogenase (OGDH) e succinato CoA ligase para produzir ATP. O NAD^+ que é necessário para esta reação é regenerado a partir de NADH via malato desidrogenase (MDH) catalisado pela reação de oxaloacetato a malato. O oxaloacetato que é exigido como substrato para esta reação é produzida pela enzima aspartato aminotransferase (AspAT). Concomitantemente, o glutamato é produzido, que é o co-substrato para a síntese de Ala. Para manter esse ciclo em funcionamento, caso as reservas de Asp e Gln esgotassem, malato é catalisado via enzima málica para formar piruvato, que pode então ser utilizado para a síntese de Ala, ou com fumarato para formar succinato. Ambas as vias podem funcionar em paralelo. Este caminho explica o papel do acúmulo de Ala durante a hipóxia, bem como a forte queda na maioria dos outros aminoácidos relacionados ao TCA.

A AlaAT é a enzima chave responsável pelo acúmulo de alanina sob hipóxia, com base em seus perfis de indução em resposta ao estresse por deficiência de O_2 (GOOD; CROSBY, 1989; MUENCH; GOOD, 1994; SOUZA; SODEK, 2003). No entanto, Souza e Sodek (2003) indicaram que o maior aumento de atividade de AlaAT acontece depois que a produção de Ala cessa, sugerindo que a função da AlaAT é desempenhada, também, durante a recuperação pós-hipóxia. Aparentemente, a produção Ala não depende unicamente da atividade da AlaAT.

Uma reação alternativa capaz de produzir Ala é catalisada pela ácido γ-aminobutírico transaminase (GABA-T), utilizando piruvato como co-substrato (ROCHA et al., 2010a).

A síntese de GABA é estimulada pela atividade da glutamato descarboxilase sob condições ácidas e contribuí para o consumo de prótons, atuando como regulador do pH citoplasmático (MIYASHITA; GOOD, 2008). A via do metabolismo do GABA envolve várias etapas e enzimas para o carbono do glutamato entrar no ciclo do ácido tricarboxílico (TCA). Sob déficit de oxigênio e na presença de Ca^{2+}/calmodulina ocorre aumento da atividade da glutamato descarboxilase (GAD), produzindo GABA e aumentando a concentração de Ca^{2+} citosólico. O GABA é então convertido a semidialdeído succínico (SSA) pela GABA-transaminase (GABA-T), produzindo simultaneamente alanina a partir de piruvato. O SSA é, então, oxidado para succinato pela semidialdeído succínico desidrogenase (SSADH) que pode ser utilizado na via do TCA, completando a via de produção do GABA (Fig. 1) (MIYASHITA; GOOD, 2008).

O sistema radicular de leguminosas é também constituído de nódulos, estruturas nos quais ocorre a fixação simbiótica de N_2 através da enzima nitrogenase. Pouco se sabe sobre o comportamento do nódulo durante o alagamento do sistema radicular. Mesmo em condições normais há uma limitação no fornecimento de oxigênio para a fixação de nitrogênio, em função de uma barreira variável à difusão de oxigênio presente nos tecidos externos do nódulo que circundam a região central infectada (HUNT et al., 1989; LAYZELL et al., 1990). Portanto, o nódulo é naturalmente hipóxico (embora levemente) e por esse motivo extremamente sensível ao alagamento em função da baixa disponibilidade de oxigênio (LIMA; SODEK, 2003; AMARANTE; SODEK, 2006).

O processo de fixação de N_2 nos nódulos é prejudicado pelo alagamento quase que imediatamente (AMARANTE; SODEK, 2006), enquanto que as raízes entram em hipóxia apenas algumas horas após a deficiência de oxigênio (SOUSA; SODEK, 2003). A falta de O_2 no nódulo é uma situação completamente diferente da de uma raiz inundada, principalmente devido ao mecanismo que envolve a leghemoglobina e a barreira de difusão aos gases, que permite ao nódulo funcionar sob baixas concentrações de O_2. Estes mecanismos possibilitam a redução do N_2 a NH_3 sem inibição da nitrogenase, que é sensível ao O_2, além do acesso ao mesmo pelas

oxidases terminais das células de rizóbio, havendo com isso o suprimento adequado de ATP necessário ao processo de fixação de N_2 (DOWNIE, 2005; UDVARDI; POOLE, 2013).

Metabolismo antioxidante sob condições de deficiência de oxigênio

O oxigênio molecular foi introduzido no ambiente há ~2,2 bilhões de anos atrás por organismos fotossintéticos e consequentemente as espécies reativas de oxigênio (EROs) passaram a fazer parte da vida aeróbica dos organismos vivos (HALLIWELL, 2006). O oxigênio, por apresentar dois elétrons desemparelhados, é considerado um radical livre capaz de aceitar elétrons, levando à formação de EROs (GILL; TUTEJA, 2010). As EROs, produtos do metabolismo, foram inicialmente consideradas como tóxicas. No entanto, as plantas produzem EROs também como moléculas de sinalização para o controle de processos como a morte celular programada, respostas a estresses abióticos, defesa contra patógenos e sinalização BLOKHINA; FAGERSTEDT, 2010a; GILL; TUTEJA, 2010).

A produção de EROs nas plantas, durante a hipóxia e também em consequência ao retorno as condições de normóxia depende da espécie, do estádio da planta, da duração e intensidade do estresse hipóxico e do tempo de recuperação (FAN et al., 1988; SCOTT et al., 1989). Entre as principais EROs, destacam-se o superóxido ($O_2^{\bullet-}$), oxigênio singleto ($^1O_2$), peróxido de hidrogênio (H_2O_2) e radicais hidroxila ($OH^{\bullet}$) (SUBBAIAH; SACHS, 2003; JACKSON; COLMER, 2005). As EROs como $O_2^{\bullet-}$ e $OH^{\bullet}$ são altamente reativas e podem alterar o metabolismo celular através de danos oxidativos nos lipídeos, proteínas e ácidos nucleicos (KUK et al., 2003; AZEVEDO NETO et al., 2006).

A produção de EROs ocorre em várias organelas, no entanto a mitocôndria e o cloroplasto estão entre as principais organelas devido à fuga de elétrons nos complexos que compõe a cadeia de transporte de elétrons, provocados por estresses como a hipóxia (MURPHY, 2009; YANG et al., 2011; ALHDAD et al., 2013).

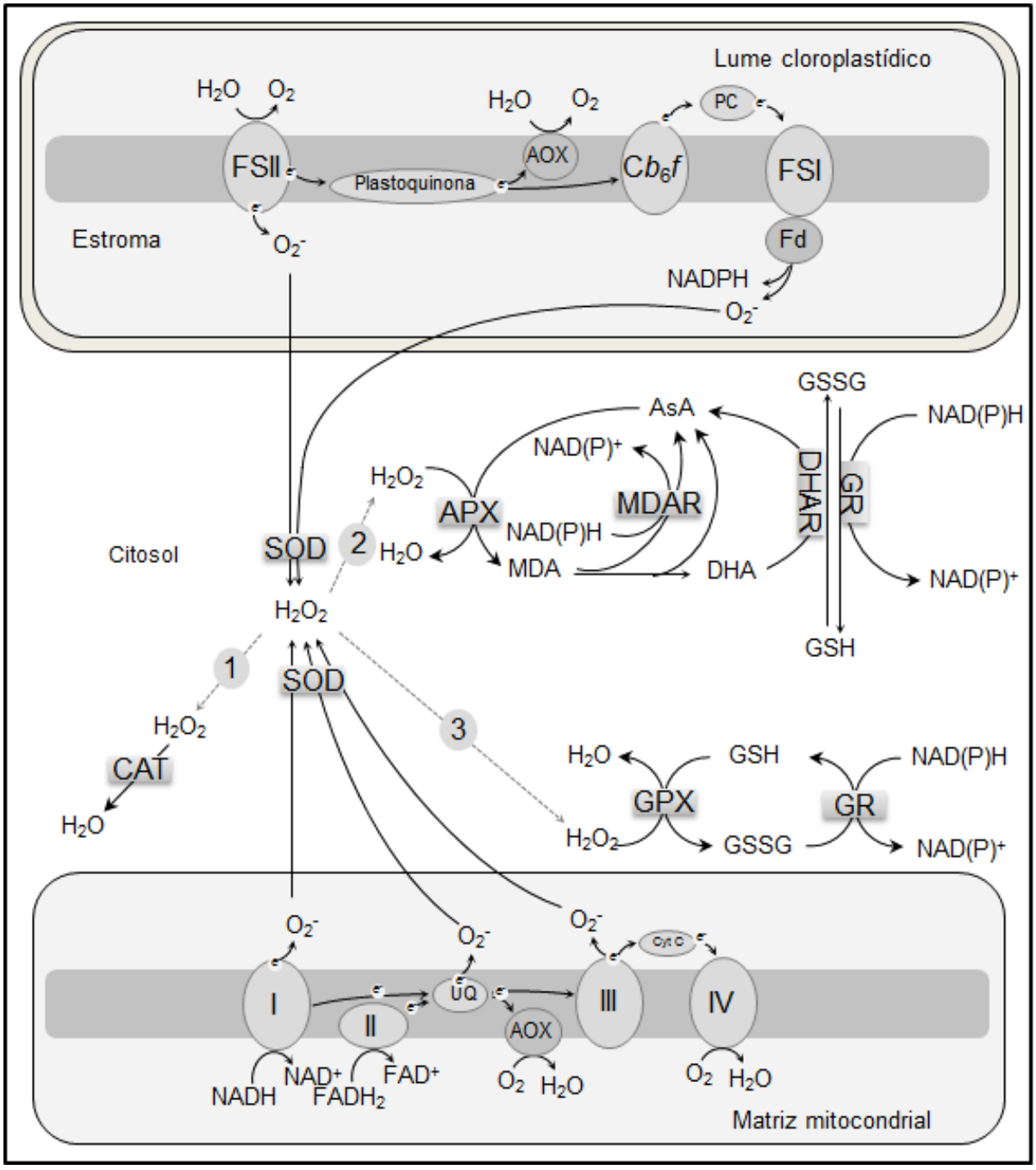

Figura 2 - Esquema representativo do sistema antioxidante e sua atuação na detoxificação de espécies reativas de oxigênio [Adaptado de Noctor e Foyer (1998) e Mittler (2002)]. Abreviações: SOD – superoxido dismutase; APX – ascorbato peroxidase; CAT – catalase; MDAR – monodehidroascorbato redutase; DHAR – dehidroascorbato redutase; GR – glutationa redutase; GPX glutationa peroxidase; AsA – ascorbato; MDA – monodehidroascorbato; DHA – dehidroascorbato; GSH - glutationa reduzida; GSSG – glutationa oxidada; O_2^- - superóxido; H_2O_2 – peroxido de hidrogênio; FSI – fotossistema I; FSII – fotossistema II; Cb_6f – citocromo; PC – plastocianina; Fd – ferredoxina; AOX - oxidase alternativa; CytC - citocromo c; UQ - ubiquinona; I, II, III e IV - complexos da cadeia de transporte de elétrons mitocondrial.

Como mecanismo utilizado para diminuir ou suprimir os efeitos danosos das EROs, as plantas desenvolveram um complexo sistema de defesa incluindo componentes não enzimáticos como glutationa, ácido ascórbico, tocoferol e sistema enzimático incluindo as enzimas superóxido dismutase (SOD), ascorbato peroxidase (APX), catalase (CAT), glutationa redutase (GR), glutationa peroxidase (GPX) e guaiacol peroxidase (GPOD), entre outras (LEE; LEE 2000; OIDAIRA et al. 2000, BLOKHINA; FAGERSTEDT, 2010a,b; GILL; TUTEJA, 2010). A SOD é a principal enzima que atua sobre o $O_2^{\bullet-}$, e sua ação enzimática resulta na formação de H_2O_2 e O_2. O H_2O_2 é então convertido a O_2 e H_2O pela ação da CAT ou em H_2O pela ação da APX (Fig. 2) (AZEVEDO NETO et al., 2006; BLOKHINA; FAGERSTEDT, 2010a,b; GILL; TUTEJA, 2010).

As peroxidases catalisam a redução do H_2O_2 a H_2O. A APX usa ascorbato como doador de elétrons no primeiro passo do ciclo ascorbato-glutationa e é considerada a mais importante peroxidase vegetal na detoxificação do H_2O_2 (NOCTOR; FOYER, 1998). GPX é menos específica ao substrato doador de elétrons, decompõe H_2O_2 pela oxidação de co-substratos como compostos fenólicos ou ascorbato. A glutationa reduzida (GSH) é responsável pela regeneração do “pool” de ascorbato produzindo glutationa oxidada (GSSG). A redução da GSSG, NADPH-dependente, é catalizada pela GR, amplamente encontrada em cloroplastos, citosol e mitocôndrias (EDWARDS et al., 1990) e o elevado nível de atividade da GR pode incrementar a razão de GSH/GSSG (GILL; TUTEJA, 2010).

Efeitos do nitrato no metabolismo vegetal

Uma das formas de remediar ou diminuir os efeitos causados nas plantas pelo alagamento, e que tem sido foco de estudo nesses últimos anos, é a aplicação exógena de nitrato (SÁNCHEZ et al., 2010; GUPTA et al., 2011; HORCHANI et al., 2011; SÁNCHEZ et al., 2011). O papel fisiológico do nitrato sob condições de hipóxia ou anóxia tem atraído a atenção de muitos pesquisadores, no entanto o entendimento de sua ação na tolerância das plantas aos efeitos do déficit de oxigênio está sujeito a muitas controvérsias, pelo fato de que em algumas espécies não foram encontrados efeitos da redução do nitrato no metabolismo energético sob hipóxia (SAGLIO et al., 1988).

Estudos *in vitro* e *in vivo* com raízes de soja não-noduladas indicaram o envolvimento da redução mitocondrial do nitrito a óxido nítrico (NO) na resposta mediada por nitrato à hipóxia (OLIVEIRA et al., 2013a,b). O nitrato, como proposto, é reduzido pela nitrato redutase (NR) formando nitrito. Sob condições de hipóxia, o nitrito pode desempenhar o papel do O_2 como aceptor de elétrons e concomitantemente ser reduzido a (NO), pela citocromo *c* oxidase (complexo IV – *COX*) levando à produção, embora limitada, de ATP (Fig. 3) (GUPTA et al., 2011). Algumas pesquisas têm mostrado que o suprimento com nitrato em tecidos radiculares anaeróbicos tem levado a um aumento do estado redox, razão NADH /NAD^+ e da carga energética (STOIMENOVA et al., 2003) através do consumo do poder redutor (NADH) gerado na glicólise e pela redução do nitrato a nitrito, o que leva a um aumento considerável da expressão dos genes e da atividade da nitrato redutase (MATTANA et al., 1996).

O nitrito também pode ser convertido a NO em uma segunda reação catalisada pela NR, no citosol (van DONGEN; LICAUSI, 2015). O NO sintetizado pode ser reduzido novamente a nitrato pelas hemoglobinas não-simbióticas da classe 1 por um mecanismo que resulta em uma oxidação adicional de NAD(P)H no citosol, contribuindo para a modulação do *status* redox e metabolismo energético de células sob hipóxia (IGAMBERDIEV et al., 2005). De forma geral, a operação conjunta dos mecanismos que controlam a homeostase do NO (síntese de NO pela cadeia respiratória e degradação pelas hemoglobinas) constitui um ciclo que possui uma importante função na manutenção do metabolismo energético das células sob hipóxia. Nesse cenário, a adubação com nitrato seria essencial para alimentar primariamente esse ciclo benéfico de *turnover* de NO.

Além do papel da homeostase do NO para tecidos sob hipóxia, essa molécula pode atuar como um importante sinalizador na resposta vegetal ao estresse. Dado que o NO pode alterar a expressão gênica (BESSON-BARD et al., 2009) e modular a atividade proteica por modificações pós-transducionais (ASTIER et al., 2012), esse sinalizador é capaz de regular o consumo de oxigênio pelos tecidos (BORISJUK et al., 2007). Dentre os transcritos modulados por NO, destacam-se aqueles relacionados a proteínas que participam da resposta vegetal ao estresse oxidativo, como a oxidase alternativa (BESSON-BARD et al., 2009) que catalisa a transferência de elétrons do ubiquinol diretamente ao oxigênio, sem passar pelos complexos III e IV e sem contribuir para a fosforilação oxidativa (VANLERBERGUE,

2013). Além de ser insensível à inibição por NO, a oxidase alternativa diminui a geração de radicais livres de oxigênio pela cadeia respiratória (VANLERBERGUE, 2013), potencialmente contribuindo para a tolerância de tecidos vegetais à injúria pós-hipóxia (SZAL et al., 2003).

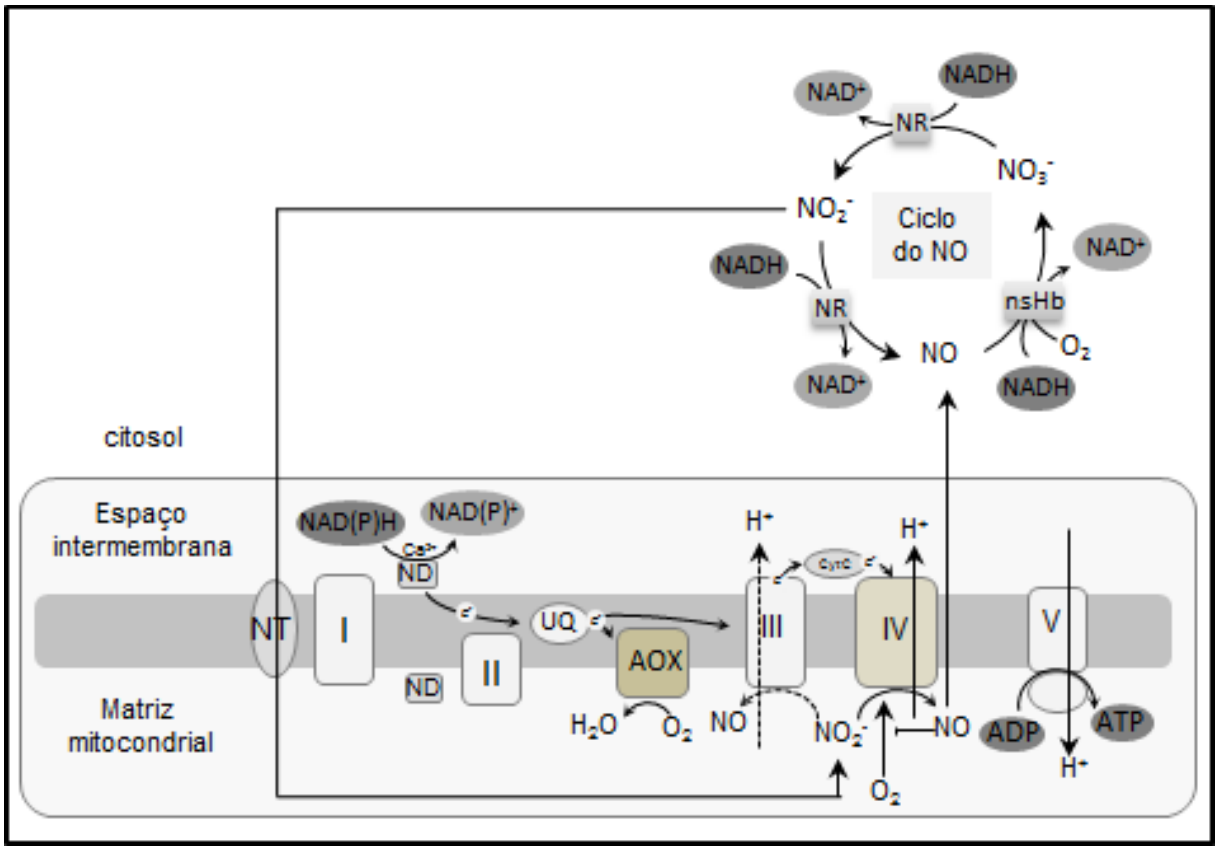

Figura 3 - Esquema representativo do ciclo do óxido nítrico mitocondrial e citosólico sob condições de deficiência de oxygênio em plantas que assimilam nitrato [Adaptado de Gupta e Igamberdiev (2011) e Gupta et al. (2011)]. Abreviações: NR – nitrato redutase; NO_3^- - nitrato; NO_2^- - nitrito; NO – óxido nítrico; nsHb – hemoglobina não simbionte; NT – transportador de nitrito; UQ – ubiquinona; AOX - oxidase alternativa; CytC - citocromo c; UQ - ubiquinona; ND - desidrogenase interna e externa; I, II, III e IV - complexos da cadeia de transporte de elétrons mitocondrial; V - ATP-sintase.

Além disso, foi reportado que raízes crescidas na presença de nitrato apresentaram um menor acúmulo de lactato e etanol que as cultivadas com amônio. Interessantemente, a intensa fermentação de raízes crescidas com amônio foi reduzida após a incubação com nitrito, um tratamento que induziu a emissão de NO em níveis semelhantes aos de raízes cultivadas com nitrato. Por fim, a produção de NO induzida pelo nitrito foi sensível a um inibidor da respiração mitocondrial,

corroborando com o envolvimento da cadeia respiratória no mecanismo de síntese de NO (OLIVEIRA et al., 2013b).

Trabalho recente demosntrou que a NR vegetal e do rizóbio (bacteriana) contribuem para a produção de NO em nódulos de *Medicago truncatula*, sugerindo a existência de um processo respiratório nitrato-NO dependente, que poderia auxiliar na manutenção do estado energético requerido para a fixação de N_2 sob condições limitantes de O_2 (HORCHANI et al., 2011). Assim, torna-se necessário estudar dentre os mecanismos que conferem tolerância da soja aos efeitos da hipóxia e pós-hipóxia, o efeito da redução e assimilação do nitrato sobre aspectos metabólicos relevantes como a atividade enzimática nas plantas, produção de metabólitos anaeróbicos, estado energético e, sistemas antioxidantes enzimáticos e não-enzimáticos de genótipos que apresentem sensibilidade diferencial ao estresse por encharcamento.

O cultivo de soja, em áreas sujeitas à inundação ou com deficiência de drenagem natural, pode ser viabilizado também por meio da identificação desses mecanismos de tolerância à deficiência de O_2 em genótipos, o que contribuirá para a caracterização e geração de cultivares mais adaptados, tornando assim, mais eficiente o atual modelo produtivo dessas áreas, que é ocupado, em grande parte, pelo monocultivo de arroz irrigado.

Embora muitos estudos têm sido realizados para elucidar os efeitos do déficit de O_2 em plantas, poucos trabalhos são relacionados com plantas noduladas, especialmente soja. Nesse contexto, visando melhor entender os efeitos ocasionados pela depleção de oxigênio no sistema radicular, o objetivo deste trabalho é caracterizar metabólitos anaeróbicos em raízes e nódulos de dois genótipos de soja que apresentam tolerância diferencial à deficiência de O_2, submetidos a períodos de hipóxia e pós-hipóxia do sistema radicular no metabolismo do carbono e nitrogênio, bem como a influência do nitrato sobre o sistema antioxidante enzimáticos e não-enzimáticos.

ARTIGO 1 - Physiologia Plantarum

Hypoxia–driven changes in glycolytic and tricarboxylic acid cycle metabolites of two nodulated soybean genotypes

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Abstract – Oxygen deprivation triggers changes at different levels of carbon and nitrogen metabolism, which may differ between tolerant and sensitive plants. The aim was to evaluate the hypoxia-induced alterations of carbon and nitrogen metabolites and its relation with alanine aminotransferase (AlaAT, EC 2.6.1.2) enzyme in nodulated soybean (*Glycine max)* genotypes. Nodulated soybean plants (Fundacep 53 RR – tolerant and BRS Macota – sensitive) were grown in vermiculite and transferred to a hydroponic system at the reproductive stage. The root system was subjected to hypoxia by continuously flushing the solution with N_2 gas for 24 or 72 h. For recovery, after 72 h in hypoxia, plants returned to normoxic conditions after transfer to vermiculite for 24 and 72 h. Root and nodule organic acids and amino acids were analysed by gas chromatography-mass spectrometry and high-performance liquid chromatography, respectively. Relative expression of *AlaAT* and AlaAT activity were also verified in both genotypes. Plants of Fundacep 53 RR and BRS Macota genotypes responded distinctly to hypoxia. Fundacep 53 RR presented higher pyruvate and lactate accumulation than BRS Macota, which is indicative of higher glycolytic and

fermentation rates in root tissues. Furthermore, Fundacep 53 RR responds more effectively on recovery by restoring pre-hypoxic levels of the metabolites. Although the amino acid composition did not differ between the genotypes, there was a clear link between glycolysis and the Krebs-cycle via increase of gene expression and activity of AlaAT allied to succinate accumulation in Fundacep 53 RR. This may represents a metabolic advantage over BRS Macota under hypoxia.

Abbreviations – Ala, alanine; AlaAT, alanine aminotransferase; ANOVA, analysis of variance; Asn, asparagine; Asp, aspartate; AspAT, aspartate aminotransferase; ATP, adenosine triphosphate; CoA, coenzyme A; DNA, deoxyribunocleic acid; GABA, γ–aminobutyric acid; GAD, glutamate decarboxylase; GC-MS, gas chromatography-mass spectrometry; Gln, glutamine; Glu, glutamate; HCl, hydrochloride acid; HPLC, high-performance liquid chromatography; MCW, methanol, chloroform and water; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide reduced; OAA, oxaloacetate; OGDH, 2-oxoglutarate dehydrogenase; OPA, *o*-phthaldialdehyde; PEPC, phosphoenolpyruvate carboxylase; PPi, pyrophosphate; RNA, ribonucleic acid; RT-PCR, real-time reverse transcription-polymerase chain reaction; SAS, statistical analysis system; Ser, serine; Suc, sucrose; SUS, sucrose synthase; TCA-cycle, tricarboxylic acid cycle.

Introduction

Waterlogging and flooding are becoming more frequent due to heavy rainfalls, a consequence of climate changes. Under these conditions oxygen supply is impaired to the roots, thus inhibiting root respiration and affecting crop growth and productivity of many species worldwide (Limami et al. 2014). Plants have shown wide variations in their ability to tolerate the limitations of oxygen concentration through a series of adaptive mechanisms to ensure its survival in an attempt to prevent or postpone the effects caused by hypoxia (Mommer et al. 2005, Bailey-Serres and Voesenek 2008, Bailey-Serres et al. 2012, Kreuzwieser and Rennenberg 2014).

Due to inhibition of the mitochondrial oxidative phosphorylation the "Pasteur effect" is triggered, leading to an increase of glycolysis to maintain ATP production and the cell viability (Summers et al. 2000). In order to limit energy consumption, plants down-regulate the synthesis of storage products such as starch and protein (Geigenberger 2003, Gupta et al. 2009). Sucrose (Suc) degradation is shifted from invertase to sucrose synthase (SUS) and

others enzymes using PPi (pyrophosphate) instead of ATP (Kumutha et al. 2008, Sairam et al. 2009, Mustroph et al. 2014b).

To keep the glycolysis running under hypoxia, fermentative enzymes are rapidly activated to continuously regenerate NAD^+ (Licausi 2011, van Dongen and Licausi 2015). Using pyruvate, fermentative reactions produce lactate via lactate dehydrogenase or ethanol via two consecutive reactions catalysed by pyruvate decarboxylase and alcohol dehydrogenase (Tadege et al. 1999, Zabalza et al. 2009). In addition, plants such as soybean (*Glycine max* L. Merrill), can accumulate alanine (Ala) under hypoxic conditions, an amino acid produced by the enzyme alanine aminotransferase (AlaAT) (Sousa and Sodek 2003). Although the synthesis of Ala is not directly associated with the recycling of NAD^+, its production helps to regulate the glycolytic flux by preventing pyruvate accumulation (Zabalza et al. 2009). Furthermore Ala can be accumulated in high concentrations even under nitrogen deficiency without causing any cell toxicity (Rocha et al. 2010a).

Hypoxic conditions also affect the activity of nitrogenase in nodules of nitrogen-fixing plants (Justino and Sodek 2013) and trigger significant changes in amino acid composition, such as a considerable reduction of glutamine content (Gln) transported in the sylem sap (Amarante and Sodek 2006).

In addition to these changes, the increase in Ala content is accompanied by a large increase of γ–aminobutyric acid (GABA), reflecting the metabolism under hypoxia (Puiatti and Sodek 1999, Thomas et al. 2005). GABA is mainly produced by glutamate decarboxylase enzyme under, which contributes to proton consumption, avoiding the deleterious effects of cytosolic acidification during hypoxia (Crawford et al. 1994).

AlaAT catalyses the reversible transamination reaction of pyruvate and glutamate into alanine and 2-oxoglutarate, linking carbon and nitrogen metabolism of plants (Rocha et al. 2010a). The connection between glycolysis and tricarboxylic acid cycle (TCA) mediated by alanine aminotransferase activity during hypoxia of *Lotus japonicus* has been proposed as a metabolic model. Under these conditions, 2-oxoglutarate resulting from the AlaAT reaction can further react within mitochondria to form succinate via 2-oxoglutarate dehydrogenase (OGDH) and succinate CoA ligase, leading to anaerobic ATP production (Rocha et al. 2010a).

It is important to emphasize that different plant species or even genotypes show significant variation in their level of tolerance to low oxygen stress in order to survive (Shingaki-Wells et al. 2014). Many of these variations are related to changes in carbon metabolism (Rocha et al. 2010a). In addition, changes in the levels of starch, glycolytic

substrates (total soluble sugars and sucrose) and fermentation metabolites (ethanol, lactate and pyruvate) were reported by Borella et al. (2014) in roots and nodules as a metabolic mechanism that differentiate soybean genotypes (Fundacep 53 RR and BRS Macota) under hypoxic conditions.

However, the mechanism described by Rocha et al. (2010a) regarding the link between AlaAT and carbon metabolism is not clear as to whether it operates in nodulated soybean genotypes where it could underlie hypoxic tolerance. Thus, the objective of this study was to investigate changes related to amino acid, TCA-cycle, glycolytic metabolites and AlaAT activity and expression in nodulated plants of two contrasting soybean genotypes under oxygen deficiency.

Material and methods

Plant material and growth conditions

Soybean plants (*Glycine max* L. Merril cv. Fundacep 53 RR and BRS Macota) were grown in a greenhouse under natural light and temperature conditions. Three plants were grown in a single plastic pot (3 l) containing vermiculite as substrate and supplied with 250 ml N-free nutrient solution twice per week (Hoagland and Arnon 1938), as described by Lima and Sodek (2003). Plants were inoculated with *Bradyrhizobium elkanii* strain SEMIA 587 (FEPAGRO) at the V1 stage and the treatments were initiated with plants at R2 stage (Flowering; early reproductive stage) described by Fehr et al. (1971). The hydroponic treatment was carried out as described by Borella et al. (2014). Plants were removed from the pots and the entire root system was carefully washed in tap water to remove the vermiculite before being transferred to 3 l pots (3 plants per pot) containing N-free nutrient solution at one-third of normal strength. The whole root system (including the nodules) was kept submersed in the nutrient solution. In the experiment, the nodulated root system was subjected to hypoxia by flushing N_2 gas for 24 h and 72 h, respectively. Oxygen concentration into the solution was monitored with an oxygen meter (Handylab OX1), demonstrating that hypoxia was rapidly reached after 6 h (Borella et al. 2014).

For recovery, after 72 h of hypoxia, plants were returned to 3 l pots containing vermiculite as substrate under normoxic conditions per 24 h and 72 h. Plants maintained in vermiculite were used as control. At harvest, four biological replicates of nodules and roots were taken up for each treatment and kept frozen (- 80ºC) until analysis.

Metabolite extraction and analysis

Low molecular weight metabolites were extracted from nodules and roots with 10 mL of methanol:chloroform:water (MCW) (12:5:3 v/v/v) per gram of plant material, then following the procedure described by Sousa and Sodek (2003). The aqueous phase resulting from MCW extraction was used for the analysis of organic acids and amino acids. Organic acids were analysed by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu QP2010plus system (Shimadzu Corporation, Tokyo, Japan) under the same conditions described by Oliveira and Sodek (2013). Individual amino acids were determined by reverse-phase high-performance liquid chromatography (HPLC) as their *o*-phthaldialdehyde (OPA) derivates based on the method described by Puiatti and Sodek (1999). The amount of total amino acids was determined using the ninhydrin method using leucine as standard (Yemm and Cocking 1955).

Alanine aminotransferase activity assay

AlaAT enzyme activity (EC 2.6.1.2) was determined in root and nodule tissues. Plant material was ground to a powder using a mortar and pestle with 50 m*M* Tris/HCl (pH 7.5) containing 1 m*M* dithiothreitol. All procedures were carried out at 4ºC. The homogenate was centrifuged at 10 000 g for 20 min, and an aliquot of the supernatant was desalted using a PD10 column (GE Healthcare, Buckinghamshire, UK). Total protein content of the enzyme extract was measured as described by Bradford (1976). The eluted protein fraction was assayed for AlaAT activity as described by Sousa and Sodek (2003). The assay (alanine $\rightarrow$ pyruvate direction) contained, in a final volume of 1.5 ml, 10 m*M* L-alanine, 5 m*M* 2-oxoglutarate, 0.1 m*M* NADH, 50 m*M* Tris/HCl (pH 7.5) and 5 units of lactate dehydrogenase (Sigma L5132) in a 1.5 ml cuvette. After the addition of extract, the cuvette was maintained in a spectrophotometer (T80 UV/VIS Spectrometer – PG Instruments) with a temperature-controlled cuvette-holder at 30ºC and the absorbance at 340 nm recorded at 10 s intervals.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

To determine changes of *AlaAT* gene expression 0.2 g of soybean roots or nodules were ground to a powder in liquid nitrogen using a pestle and mortar. RNA was extracted after

addition of 1 ml of TRIzol® reagent (Invitrogen, Carlsbad, USA) following the manufacturer's recommendations. The RNA extracts were stored at -80ºC until further processing. For the synthesis of cDNA, 2.0 µg of total RNA was first treated with DNA-free DNase I (Invitrogen, Carlsbad, USA), to remove DNA contamination. cDNA was synthesized using oligo(dT) and SuperScript™ III reverse transcriptase kit (Invitrogen, Carlsbad, USA). Subsequently, cDNA was used as template for a real time – polymerase chain reaction (RT-PCR) amplification using power SYBR®- green master mix (Applied biosystems, Carlsbad, USA), Actin was used as reference gene. The following primers were used: *GmAlaAT1* forward sequence: CCCCAAGGTTCTGAAATGTGA; and reverse sequence: TTGCAAATTCTGGGCAAGTGT; *GmAlaAT2* forward sequence: TTCCAGTCCCACAATACCCAC and reverse sequence: CACCAAGCAGAGCAATTGTTG; *Actin* forward sequence: TAATGAGCTTCGTGTGGCCC and reverse sequence: GCCTCCGTCAACAGAACTGG.

Statistical analysis

Each treatment consisted of three replicates and each replicate consisted of one pot containing three plants (material pooled), in a fully randomized design. The data were analysed by one-way analysis of variance (ANOVA). When *F* was significant the treatments means for each genotype or the genotypes for each treatment were compared by Tukey test ($p \leq 0.05$). Statistical analyses were performed using the SAS 8.0 statistical software program (SAS Institute Inc. Cary, NC, USA).

Results

Glycolysis and tricarboxylic acid cycle metabolites under hypoxia

Changes in pyruvate, lactate and TCA cycle organic acids content (citrate, 2-oxoglutarate, succinate, fumarate and malate) were determined in roots (Fig. 1) and nodules (Fig. 2) of two genotypes, Fundacep 53 RR (tolerant) and BRS Macota (sensitive). A marked increase in pyruvate (Figs. 1A; 2A) and lactate content (Figs. 1B; 2B) were observed in roots and nodules of both genotypes after 24 and 72 h of hypoxia. Differences between both genotypes regarding lactate and pyruvate accumulation were detected, with higher (~ 50%)

levels of these metabolites in roots and nodules of Fundacep 53 RR under hypoxia in comparison with BRS Macota (Figs. 1A; B). With the subsequent return to normoxia, the content of these metabolites in Fundacep 53 RR decreased to the pre-hypoxic levels (control), while in BRS Macota lactate and pyruvate levels remained higher even after 72 h of recovery in roots (Figs. 1A; B) and in nodules decreased levels were observed although they did not reach the control (Figs. 2A; B).

Citrate (Fig. 1C) and 2-oxoglutarate (Fig. 1D) levels did not change under hypoxic conditions in roots of both genotypes, except for citrate at 24 h in roots of Fundacep 53 RR. However, in roots of BRS Macota plants, citrate (Fig. 1C) and 2-oxoglutarate levels increased under recovery (Fig. 1D). In nodules of both genotypes, there was a gradual increase in citrate (Fig. 2C). Oxoglutarate levels increased in BRS Macota under hypoxia and post-hypoxia, but this was not observed in Fundacep 53 RR (Fig. 2D).

The succinate content increased with 24 h of hypoxia in roots of Fundacep 53 RR and decreased to pre-hypoxic levels after recovery (Fig. 1E). On the other hand, in roots of BRS Macota there was no significant increase in the content of this metabolite (Fig. 1E). However, a substantial increase of succinate levels was observed in hypoxic nodules of both genotypes and they remained higher than control levels up to the end of the recovery period (Fig. 2E).

After 24 h, hypoxia induced a decrease of fumarate content in BRS Macota roots compared to the control, whereas a reduction of the levels of this metabolite in Fundacep 53 RR roots was observed only after 48 h of hypoxia (Fig. 1F). Malate content was increased by 24-h hypoxia only in Fundacep 53 RR roots (Fig. 1G). However, at 72 h of hypoxia, root malate levels decreased in both genotypes when compared to the control (Fig. 1G). In nodules of both genotypes, fumarate (Fig. 2F) and malate contents (Fig. 2G) gradually increased with the hypoxic treatment and subsequent recovery.

Effects of hypoxia on amino acid content

The amino acid composition of roots and nodules during hypoxia and after return to normoxia is shown in Figure 3. Initially (normoxia), asparagine was the most abundant amino acid in both genotypes and tissues, representing 22.37% (Fig. 3A) and 30.05% (Fig. 3B) in roots and 27.37% (Fig. 3C) and 42.44% (Fig. 3D) in nodules of Fundacep 53 RR and BRS Macota, respectively. During hypoxia, the amino acid composition changed substantially, with the most noteworthy variations being the reduction of asparagine to very low levels, a marked increase in the content of GABA and a more discrete increase in alanine. In both

genotypes, alanine reached proportions of approximately 15% in roots and 20% in nodules, while GABA reached 55% in roots and 37% in nodules with 72 h of hypoxia. Over the same period of hypoxia, the proportion of other major amino acids (Asp, Glu, Asn and Gln) were reduced to less than 5% in roots and nodules of both genotypes (Fig. 3).

When normoxia was re-established, the concentration of Ala and GABA reduced substantially over 24 and 72 h of recovery in roots and nodules for both genotypes, almost reaching pre-hypoxic percentages (Fig. 3). Simultaneously, an increased proportions of Asp, Glu, Gln and especially Asn were observed during the recovery period.

Total soluble amino acids in roots increased with hypoxia, reaching 60% and 56% increments compared to their controls in Fundacep 53 RR and BRS Macota plants, respectively, at 72 h of hypoxia (Fig. 4A). In nodules there was no variation in amino acid content during the whole experiment in Fundacep 53 RR. On the other hand, in BRS Macota nodules, the total amino acid concentration was reduced during hypoxia, and it was not re-established to control levels even after 72 h of recovery (Fig. 4B).

Effects of hypoxia on the activity and gene expression of AlaAT

The activity of AlaAT in roots (Fig. 5) increased substantially after 72 h of hypoxia in both genotypes, and was re-established to the pre-hypoxic levels of activity at 72 h of recovery only in roots of Fundacep 53 RR. After 72 h of hypoxia, AlaAT activity in roots of Fundacep 53 RR was approximately twice that of BRS Macota. Similarly, after the same period of hypoxia, the relative expression of *AlaAT1* was 2-fold higher in roots of Fundacep 53 RR in comparison with BRS Macota (Fig. 5C). While the expression of *AlaAT1* was induced by hypoxia in Fundacep 53 RR roots (Fig. 5C), the relative expression of *AlaAT2* was reduced under hypoxic conditions in both genotypes (Fig. 5E). However, it is noteworthy that, when comparing both genotypes during hypoxia, *AlaAT2* expression was higher in Fundacep 53 RR roots (Fig. 5E).

The activity of AlaAT in Fundacep 53 RR nodules increased at 72 h of hypoxia and remained elevated up to 72 h of recovery. In BRS Macota nodules, AlaAT activity significantly increased only at 24 h of recovery (Fig. 5B). The relative expression of the gene *AlaAT1* was rather variable and with one exception differences were not statistically significant throughout the hypoxic and recovery treatments in nodules of both genotypes (Fig. 5D). *AlaAT2* expression on the other hand was inhibited under hypoxic conditions and its expression returned to control levels in both genotypes during recovery (Fig. 5F).

Discussion

Several adaptive responses are initiated by plants as a mechanism to alleviate the consequences of oxygen deficiency during flooding or waterlogging (Bailey-Serres and Voesenek 2008, Bailey-Serres et al. 2012, Kreuzwieser and Rennenberg 2014). In particular, there are many metabolic alterations that may confer tolerance of certain species to hypoxic conditions. However, the specific changes and the extent to which they occur may differ among genotypes subjected to hypoxia as observed in nodulated soybean genotypes studied here, Fundacep 53 RR and BRS Macota (Figs. 1-5).

Previous work of our group has demonstrated differences between Fundacep 53 RR and BRS Macota regarding carbohydrate and fermentative metabolism (Borella et al. 2014). Fundacep 53 RR is a genotype that is more tolerant to waterlogging than BRS Macota. The higher accumulation of lactate and pyruvate in roots of Fundacep 53 RR (Figs. 1A; B) may be a characteristic of tolerance. This higher production of pyruvate was associated with the induction of *AlaAT1* gene (Fig. 5C) and increased AlaAT enzyme activity (Fig. 5 C), leading to the production of Ala (Fig. 3) and 2-oxoglutarate (Fig. 1D), under hypoxia. The 2-oxoglutarate may be further catalysed within the mitochondria leading to increased succinate production (Fig. 1E). The absence of an increase of succinate suggests the mechanism proposed by Rocha et al. (2010a), may not be functioning in roots of BRS Macota under hypoxia.

Increased pyruvate production can be explained by glycolysis activation (Pasteur effect) leading to a production of 2 ATP per mol of glucose and the cytosolic NAD^+ recycled from NADH via fermentation reactions. Lactate production in both roots and nodules increase significantly (Figs. 1B; 2B) to maintain the redox reactions under hypoxia in both genotypes, via lactate dehydrogenase activity (Licausi 2011) as observed by Borella et al. (2014). After the subsequent return to normoxia, lactate content decreased to the levels of the control in roots (Fig. 1B) and nodules (Fig. 2B) in Fundacep 53 RR, while in BRS Macota they did not return to control levels even after 72 h of recovery in neither of these tissues.

Various morphological and physiological changes also occur in plants in response to flooding as a mechanism to reduce the metabolic requirement for energy and increase the oxygen availability to the submerged tissues (Justin and Armstrong 1987). All these adjustments are described as the low oxygen escape syndrome (Bayley-Serres and Voesenek 2008) and underlie an important survival strategy to hypoxia (Rocha et al. 2010a), which were evident in the biochemical changes that clearly influence the metabolism of carbon and

nitrogen, mainly in roots (Figs. 1; 2; 3) that could drive a hypoxic tolerance in Fundacep 53 RR in comparison with BRS Macota.

A high carbon demand is required to maintain glycolysis operating during hypoxic conditions (Kumutha et al. 2008, Sairam et al. 2009). Apparently, plants show a down-regulation in the synthesis of storage products such as starch and protein when the energy demand is high (Gupta et al. 2009) thus increasing the carbon flux to maintain glycolysis. As reported by Borella et al. (2014), roots of the Fundacep 53 RR genotype presented a decreased of the starch pool, while an accumulation of starch occurred in BRS Macota at 72 h of hypoxia.

As reported by Borella et al. (2014), lactate, ethanol levels and the enzymes activities of fermentative pathways changed in a similar way. Collectively, pyruvate and lactate fluctuations levels suggest a higher glycolytic activity in Fundacep 53 RR under hypoxia and a faster recovery during post-hypoxia stress.

Moreover, glycolysis is important for providing substrate for amino acids synthesis (Shingaki-Wells et al. 2011). Pyruvate is used in amino acid metabolism for Ala production via alanine aminotransferase reaction (Sousa and Sodek 2002), which, concomitantly produces 2-oxoglutarate. This in turn can be converted into succinyl-CoA, by 2-oxoglutarate dehydrogenase leading to a NADH production. Succinyl-CoA can be further metabolized to produce ATP and succinate. The NAD^+ required for succinyl-CoA production is provided by via malate dehydrogenase activity in a reverse reaction of the TCA-cycle, from oxaloacetate to malate (Rocha et al. 2010a). Succinate is accumulated within the mitochondria since the enzyme which catalyses the following reaction, succinate dehydrogenase is strongly inhibited due to saturation of the ubiquinone pool (Rocha et al. 2010a), leading to a decrease in fumarate production, in both soybean genotypes (Fig. 1F).

Our findings clearly demonstrate to be in agreement with the mechanism proposed in roots of *Lotus* by Rocha et al. (2010a), since there was accumulation of succinate in roots of Fundacep 53 RR with 24 h of hypoxia that could lead to ATP production and as well as malate accumulation which would help to oxidize NADH produced via OGDH by converting 2-oxoglutarate to further produce succinate. On the other hand, this mechanism did not appear to be operating in roots of BRS Macota, opening the possibility it may underlie the greater tolerance of Fundacep 53 RR to waterlogging.

Oxaloacetate can be replaced via carboxylation of phosphoenolpyruvate by phosphoenolpyruvate carboxylase (PEPC) or via aspartate aminotransferase (AspAT). Indeed, there is evidence that aspartate is an important source of N for alanine formation in a coupled

reaction involving AspAT and AlaAT (Streeter and Thompson 1972, Vanlerberghe et al. 1991, Good and Muench 1993). Malate can also be converted to pyruvate by malic enzyme (Miyashita et al. 2007, Rocha et al. 2010a), which might explain, in part, the decreased accumulation of this metabolite in roots after 72 h of hypoxia.

Thus, there is a correlation between the nitrogen and carbon status in roots of Fundacep 53 RR and prolonged survival ability under hypoxic conditions. Especially in roots, most amino acids derived from the TCA-cycle during the flooding decrease (Fig. 3), for example glutamine, asparagine, glutamate and aspartate, as also reported by Mustroph et al. (2014a) and Narsai et al. (2011), whereas Ala and GABA increase in roots. In addition, BRS Macota accumulated lower amounts of TCA cycle metabolites in comparison with Fundacep 53 RR (Fig. 1) possibly due to a lower rate of entrance of oxaloacetate and 2-oxoglutarate within the TCA cycle.

The accumulation of Ala and GABA under hypoxic conditions has been reported by many authors in several species (Muench et al. 1998, Muench and Good 1994, Ricoult et al. 2006, Narsai et al. 2009), including soybean (Sousa and Sodek 2002, Rocha et al. 2010a; 2010b, Oliveira et al. 2013). GABA accumulation may involve increased GAD (glutamate decarboxylase) activity but reduced GABA shunt activity may also play a part.

The GABA shunt involves several steps whereby GABA is formed by decarboxylation of glutamate followed by the convertion of GABA to succinic semialdehyde and final re-entry into the TCA cycle at succinate (Shelp et al. 1999). The final reaction, oxidation of succinic semialdehyde to succinate, is unlikely to be important under hypoxia since it requires NAD^+ and proceeds at high pH (Shelp et al. 1995, Narayan and Nair 1990). This may explain the accumulation of GABA in plants under hypoxia (Kinnersley and Turano 2000). However, since a small portion of alanine accumulated in hypoxic roots of *Arabidopsis* has been shown to be formed via the GABA shunt (Miyashita and Good 2008) it may not be totally inactive during hypoxia.

Although the production of Ala does not directly involve NADH oxidation, it is suggested that its synthesis indirectly helps to maintain glycolysis running, leading to ATP production and by controlling the levels of pyruvate, as reported in *Lotus* (Rocha et al. 2010a). Associated reactions, such as the reduction of OAA (product of the metabolic sequence Asp $\rightarrow$ Glu $\rightarrow$ Ala) to malate, may regenerate the NAD^+ needed to keep glycolysis running and thereby replace pyruvate consumed in Ala formation. Moreover, AlaAT by regulating pyruvate concentrations prevents the activation of oxygen consumption through the

alternative oxidase within mitochondria under hypoxia (Gupta et al. 2009, Zabalza et al. 2009).

The increase in Ala production is also related to an increase in AlaAT activity (Figs. 5A; B), concomitant with the relative expression of the gene *AlaAT1* that encodes for AlaAT (Fig. 5C) in the Fundacep 53 RR genotype. Its activity, as reported by Rocha et al. (2010a), played an important role linking carbon and nitrogen metabolism, explaining the higher accumulation of TCA metabolites in roots of Fundacep 53 RR. In soybean, four genes were found to encode the enzyme AlaAT. However they are divided into two subfamilies GmAlaAT1 and GmAlaAT2, with GmAlaAT1 as being the most responsive in roots of soybean (Rocha et al. 2010b) and *Arabidopsis* under hypoxia (Mustroph et al. 2014a). In *Medicago truncatula*, AlaAT gene expression and alanine accumulation in the embryo axis was also found to contribute to anoxia stress tolerance (Ricoult et al. 2005).

A possible reason for the similar accumulation of Ala in both genotypes (Fig. 3), may be related to a higher transport of Ala through xylem sap supported by higher activity and gene expression of AlaAT (Fig. 5) in Fundacep 53 RR in comparison with BRS Macota. The role of AlaAT enzyme is not limited to hypoxic conditions. It appears to be very important during the recovery period (Figs. 5A; B) and even under very low gene transcription (Fig. 5C) the enzyme activity remains high. This high post-hypoxia activity of AlaAT has an important physiological significance due to enzyme's ability to catalyse the reverse reaction, from Ala to pyruvate (Figs. 5A; B), leading to a decrease of the Ala levels (Fig. 3) that accumulated during hypoxia thereby providing pyruvate for the Krebs cycle and nitrogen for the formation of other nitrogen compounds (Sousa and Sodek 2003, Rocha et al. 2010b).

In addition to the importance of AlaAT upon return to normoxic conditions, the faster recovery of Fundacep 53 RR over BRS Macota with regard to the return to normal levels of some metabolites, especially pyruvate and lactate, indicating a more responsive to the effects of post-hypoxia.

Despite the differences in root metabolism between genotypes that may underlie Fundacep 53 RR being more tolerant to low oxygen, changes in nodules were also important and appeared to be more related to hypoxic and recovery effects than genotype-specific. In addition, changes in nodules reported by Rocha et al. (2010a) were not reported to determine tolerance in *Lotus*, in agreement with our results. The tolerance mechanism in soybean appears to be more related to its capacity of changing the metabolism in roots that counteract the effects of low oxygen in order to survive.

Conclusions

Genotypes Fundacep 53 RR and BRS Macota respond distinctly to hypoxia. Fundacep 53 RR has a higher glycolytic rate and more efficient fermentation. Although the amino acid composition did not differ between the genotypes, there is clearly a link between glycolysis and the TCA-cycle via AlaAT enzyme which leads to succinate accumulation and consequently an increased ATP gain compared to BRS Macota. Furthermore, Fundacep 53 RR responds more effectively to recovery by restoring pre-hypoxic levels of the metabolites.

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Author contributions

All persons designated as authors qualify for authorship.

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Figure legends

Fig. 1. Pyruvate (A), lactate (B), citrate (C), 2-oxoglutarate (D), succinate (E), fumarate (F) and malate (G) content in roots of soybean genotypes (Fundacep 53 RR and BRS Macota) under hypoxia and recovery (following 72 h hypoxia) conditions. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for each genotype. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between genotypes for each treatment. Black arrows represent the flow of metabolites in hypoxic conditions and gray arrows represent the flow under normoxic conditions. Values represent the mean ± SE ($n = 3$).

Fig. 2. Pyruvate (A), lactate (B), citrate (C), 2-oxoglutarate (D), succinate (E), fumarate (F) and malate (G) content in nodules of soybean genotypes (Fundacep 53 RR and BRS Macota) under hypoxia and recovery (following 72 h hypoxia) conditions. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for each genotype. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between genotypes for each treatment. Black arrows represent the flow of metabolites in hypoxic conditions and gray arrows represent the flow under normoxic conditions. Values represent the mean ± SE ($n = 3$).

Fig. 3. Amino acid composition (mol %) in roots (A and B) and nodules (C and D) of soybean genotypes (Fundacep 53 RR and BRS Macota) under hypoxia and recovery conditions. ($n = 3$).

Fig. 4. Total soluble amino acids in roots (A) and nodules (B) of soybean genotypes (Fundacep 53 RR and BRS Macota) under hypoxia and recovery (following 72 h hypoxia) conditions. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for each genotype. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between genotypes for each treatment. Values represent the mean ± SE ($n = 3$).

Fig. 5. Alanine aminotransferase activity (AlaAT) in roots (A) and nodules (B) and relative expression of *AlaAT1* and *AlaAT2* isoforms in roots (C and E) and nodules (D and F) of soybean genotypes (Fundacep 53 RR and BRS Macota) under hypoxia and recovery (following 72 h hypoxia) conditions. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for each genotype. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between genotypes for each treatment. Values represent the mean ± SE ($n = 3$).

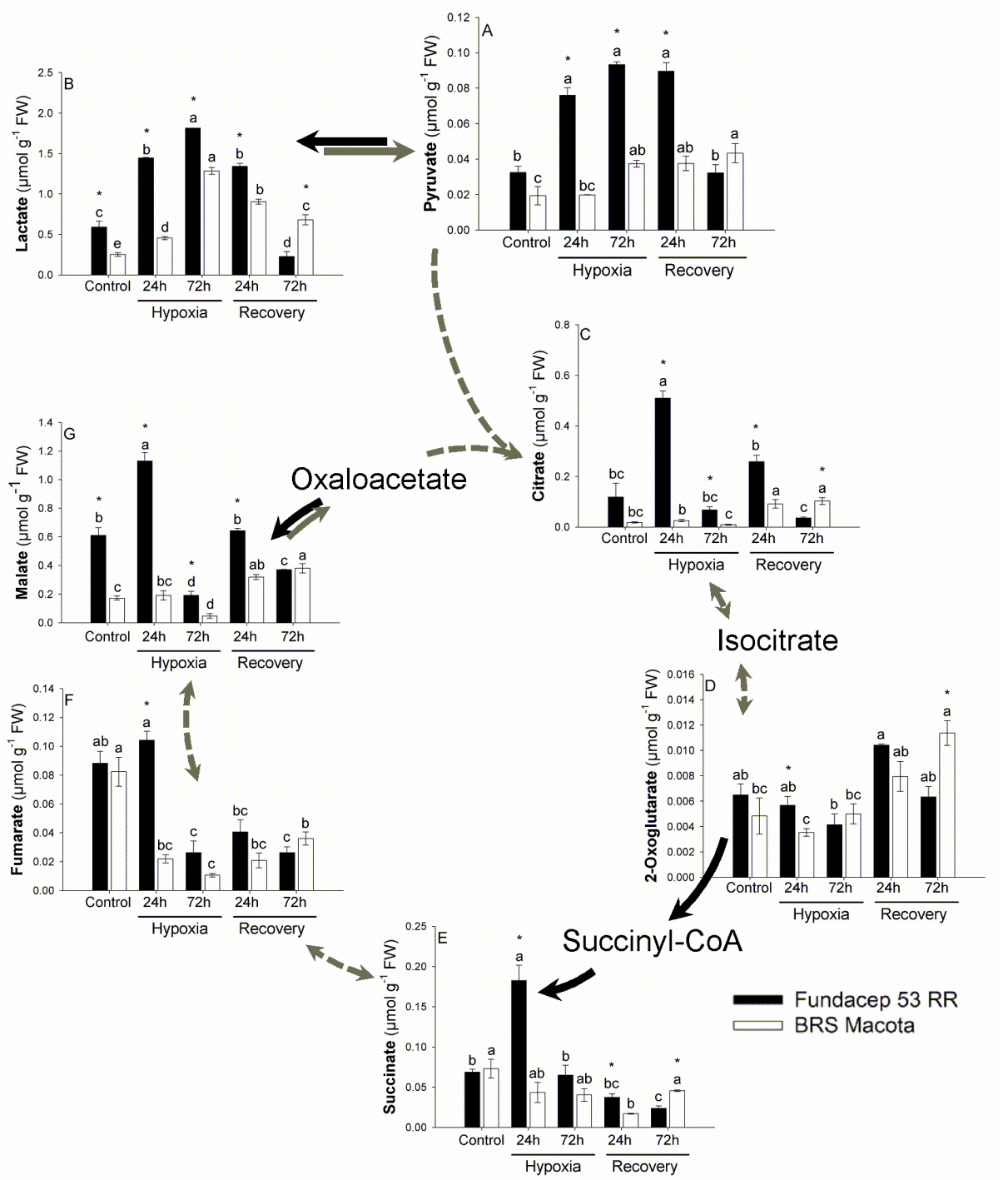
A
Pyruvate (µmol g-1 FW)
B
Lactate (µmol g-1 FW)
C
Citrate (µmol g-1 FW)
D
2-Oxoglutarate (µmol g-1 FW)
E
Succinate (µmol g-1 FW)
F
Fumarate (µmol g-1 FW)
G
Malate (µmol g-1 FW)
Control
24h
72h
Hypoxia
Recovery
Oxaloacetate
Isocitrate
Succinyl-CoA
Fundacep 53 RR
BRS Macota

Figure 1

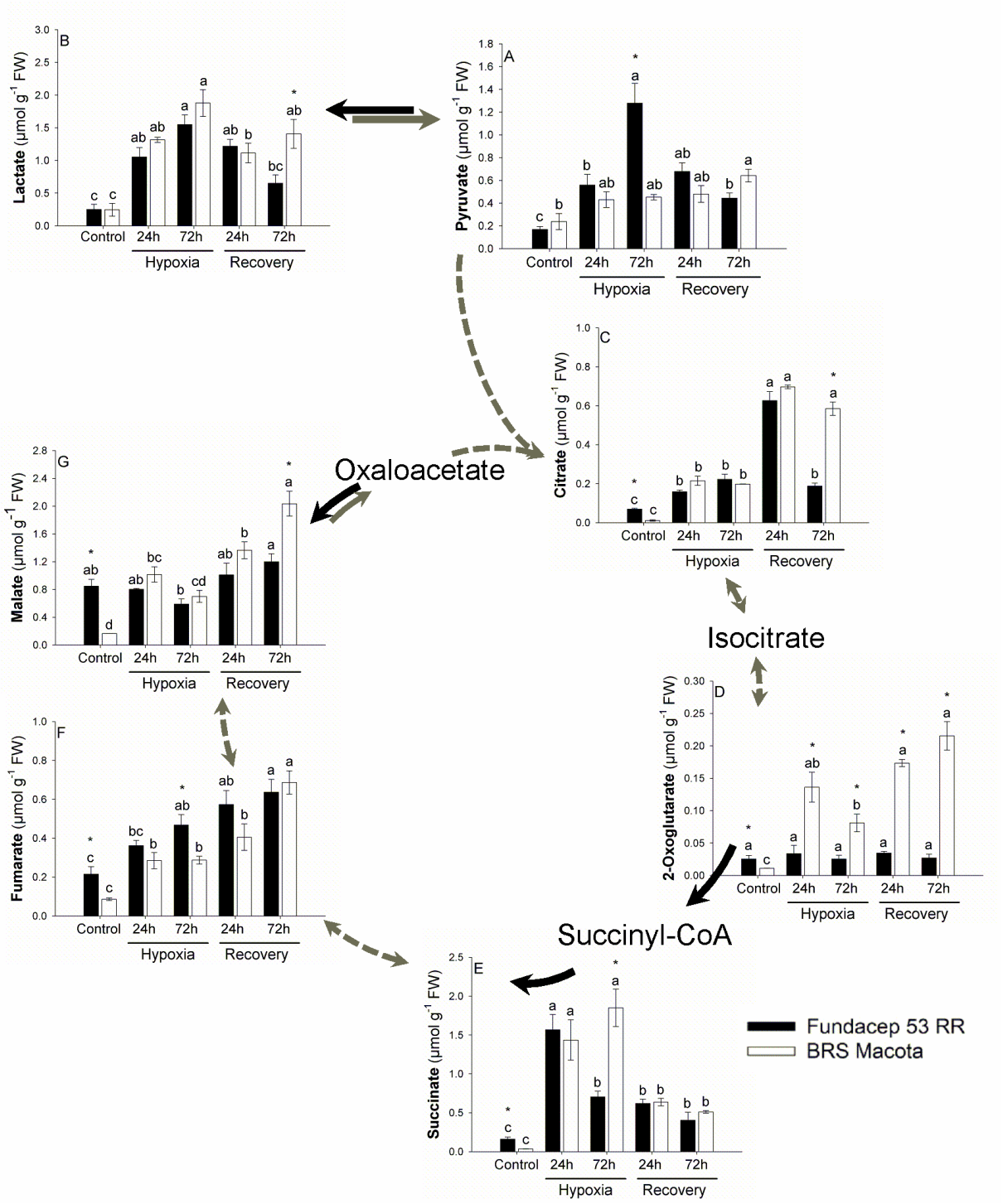
B
Lactate (µmol g-1 FW)
A
Pyruvate (µmol g-1 FW)
C
Citrate (µmol g-1 FW)
Oxaloacetate
G
Malate (µmol g-1 FW)
Isocitrate
D
2-Oxoglutarate (µmol g-1 FW)
F
Fumarate (µmol g-1 FW)
Succinyl-CoA
E
Succinate (µmol g-1 FW)
Control
24h
72h
Hypoxia
Recovery
Fundacep 53 RR
BRS Macota

Figure 2

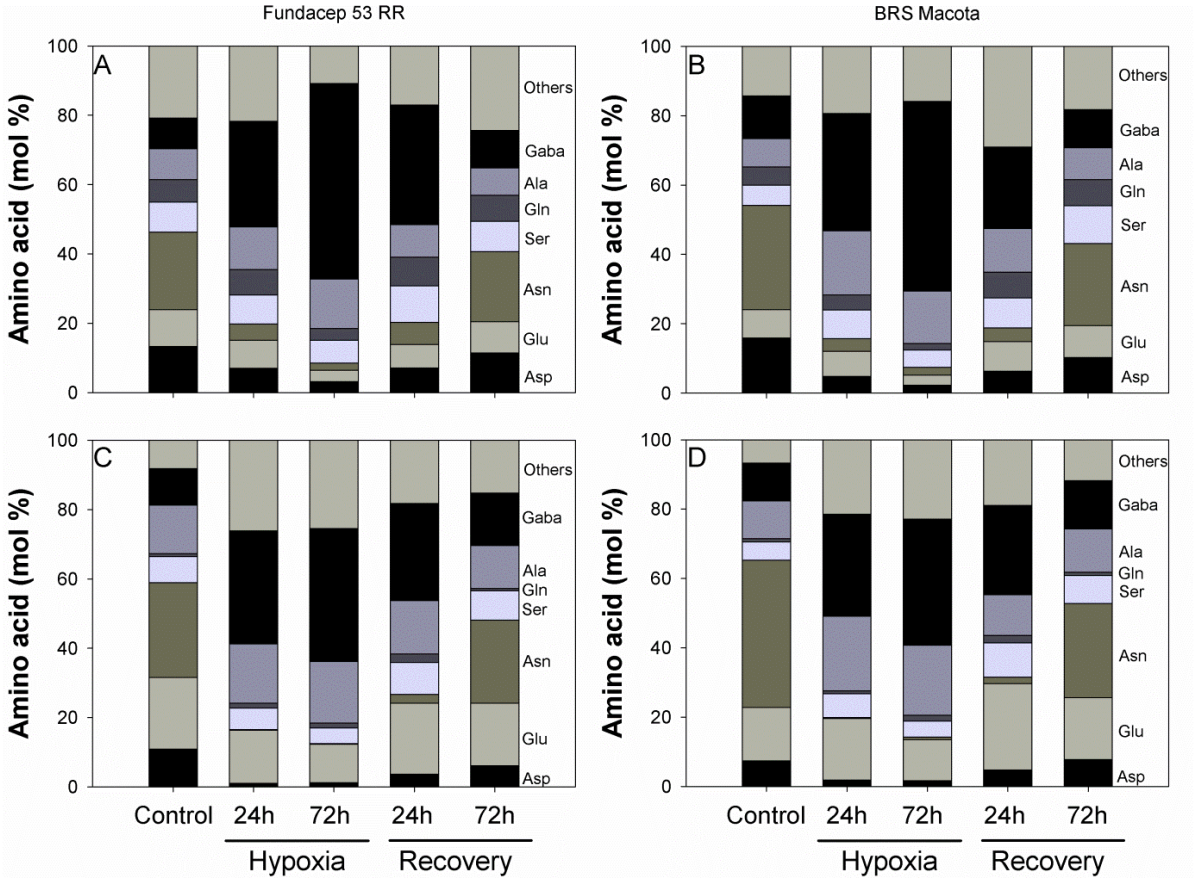
Fundacep 53 RR
BRS Macota
A
B
C
D
Amino acid (mol %)
100
80
60
40
20
0
Others
Gaba
Ala
Gln
Ser
Asn
Glu
Asp
Control
24h
72h
24h
72h
Hypoxia
Recovery

Figure 3

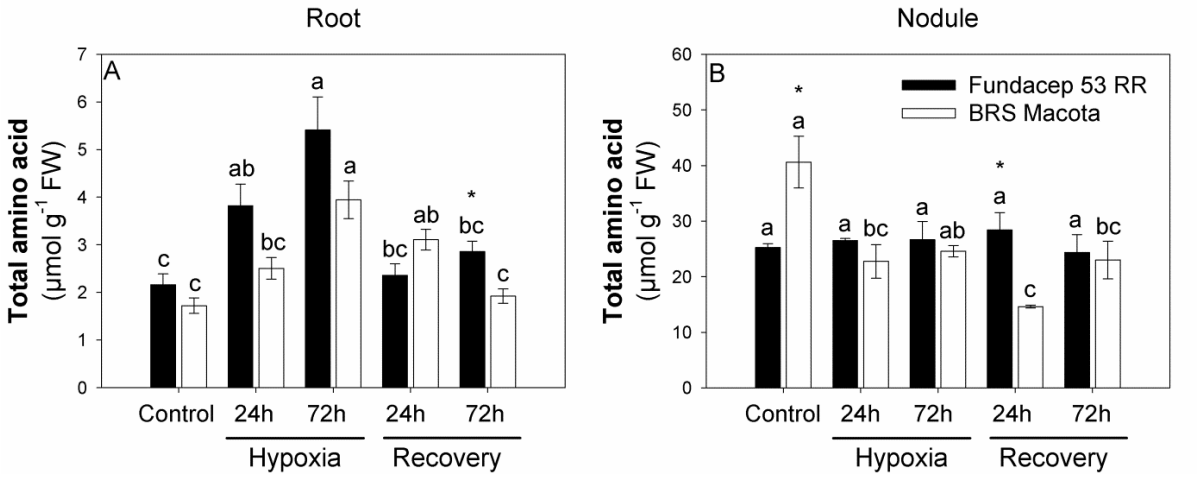
Root
Nodule
A
B
Total amino acid
(µmol g-1 FW)
Fundacep 53 RR
BRS Macota
Control
24h
72h
24h
72h
Hypoxia
Recovery

Figure 4

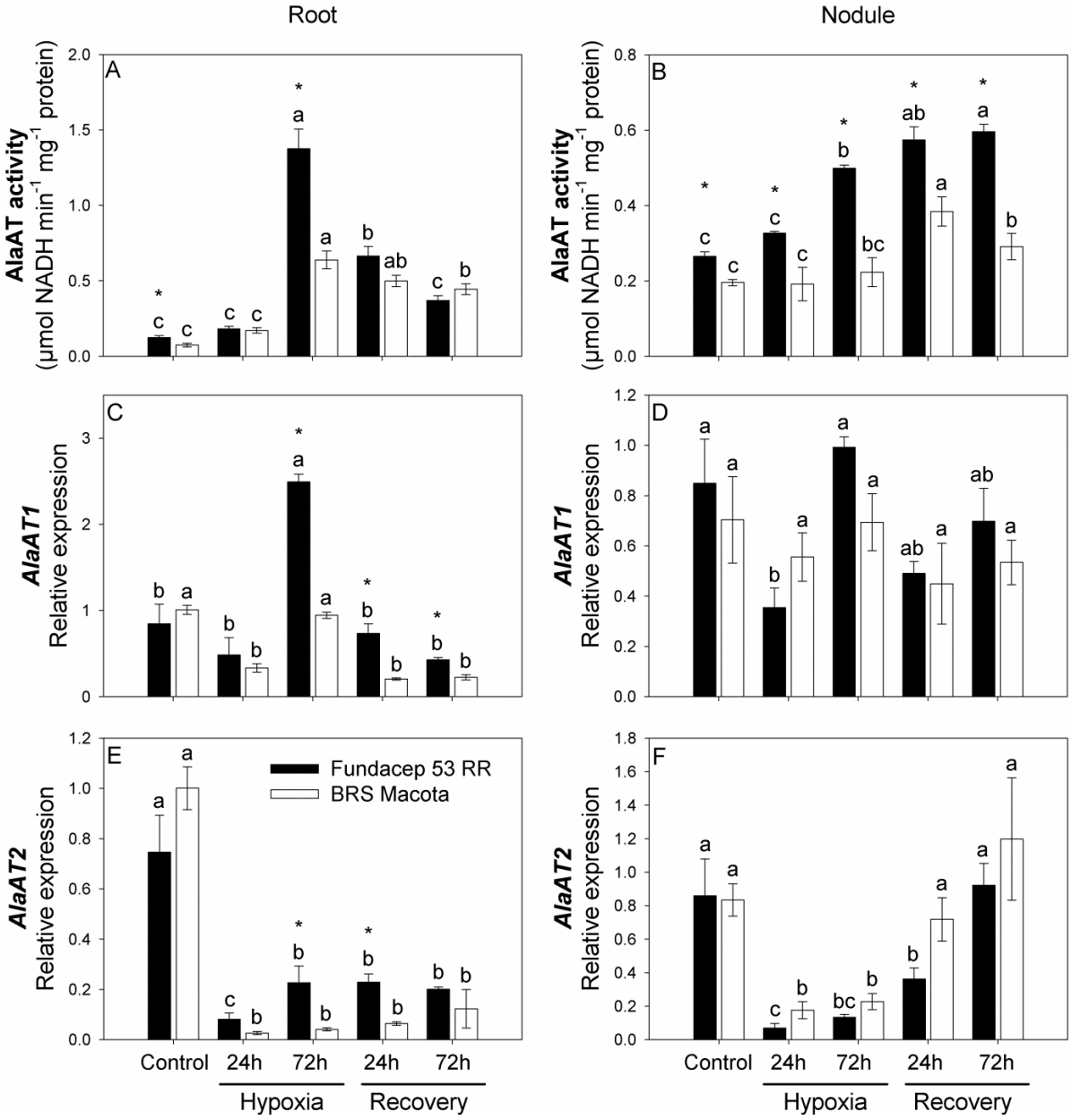
Root
Nodule
A
B
C
D
E
F
AlaAT activity
(µmol NADH min-1 mg-1 protein)
AlaAT1
Relative expression
AlaAT2
Relative expression
Fundacep 53 RR
BRS Macota
Control
24h
72h
24h
72h
Hypoxia
Recovery

Figure 5

ARTIGO 2 – Acta Physiologiae Plantarum

Antioxidant system is modulated by nitrate in soybean plants during and after hypoxic stress

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Abstract – Waterlogging is an environmental stress which causes oxygen deprivation in plants and lead to electron scape from the mitochondrial and chloroplast electron transport chain, producing reactive oxygen species (ROS). Although exogenous nitrate supply has been reported to promote beneficial effects in several plant species, only primary carbon and nitrogen have been investigated under hypoxia. In this work, we compared nitrate-supplied plants (non-nodulated) with non-nitrate-supplied plants (nodulated) in order to verify whether nitrate exerts beneficial effects on the antioxidant system under hypoxia. Antioxidant enzymatic activities, ascorbate redox state and ROS levels were analysed in roots and leaves of two soybean (*Glycine max* L. Merril) genotypes at reproductive stage in presence (non-nodulated) and absence of nitrate (nodulated) during and after hypoxia in an experiment carried out in a hydroponic system. Antioxidative system was strongly induced in roots of nitrate-supplied plants of both genotypes, with high activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and guayacol peroxidase (GPOD), as well as increased ascorbate reduced and ascorbate redox state and decreased ROS production under hypoxia and recovery, while in leaves of nodulated and non-

nodulated plants a slight increase on antioxidant system was observed. Furthermore, the results did not show tolerance differences between the genotypes. Nitrate exerts beneficial effects in soybean plants under hypoxic conditions and consequent recovery by inducing the antioxidant system mainly in roots, to cope possible oxidative damage caused by ROS production.

Keywords: *Glycine max*, hypoxia, oxidative stress, antioxidant system.

Abbreviations

ANOVA	analysis of variance
APX	ascorbate peroxidase
AsA	ascorbate
ATP	adenosine triphosphate
CAT	catalase
CDNB	2,4-dinitrochlorobenzene
COX	citocromo *c* oxidase
DHA	dehidroascrobate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GPOD	guayacol peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
MDA	Malondialdehyde
N	nitrogen
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced
NADPH	nicotinamide adenine dinucleotide phosphate reduced
NBT	nitroblue tetrazolium
NO	nitric oxide
NO_3^-	nitrate
O_2	oxygen

$O_2^{\bullet-}$	superoxide anion
PVPP	polyvinilpolypyrrolidone
ROS	reactive oxygen species
SAS	statistical analysis system
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
ε	molar extinction coefficient

Author contributions

All persons designated as authors qualify for authorship.

Introduction

Waterlogging is the major environmental stress which causes oxygen deprivation to the plant roots (Bailey-Serres and Colmer 2014; Limami et al. 2014) due to the low oxygen diffusion in the soil, 10^4 times lower in water than in air (Armstrong et al. 1994). Under hypoxia, oxygen concentration in the cells becomes too low to support aerobic respiration, resulting in the inhibition of mitochondrial oxidative phosphorylation and decreasing ATP production (Bailey-Serres and Voesenek 2008; Bailey-Serres et al. 2012; van Dongen and Licausi 2015), leading to a severe constraint on crop growth and productivity of plants in many regions worldwide (Jackson and Colmer 2005; Bayley-Serres and Colmer 2014; Limami et al. 2014).

Oxygen acts as terminal electron acceptor within the aerobic mitochondrial metabolism allowing electron transport along the chain of inner mitochondrial membrane-associated carriers and proton extrusion to create the electrochemical gradient responsive for driving the ATP synthesis (Noctor et al. 2007; Blokhina and Fagerstedt 2010a). In addition, photosynthesis takes place in chloroplasts, which contain a highly organized thylakoid membrane system that harbours all components of the light-capturing photosynthetic apparatus and provides all structure properties for optimal light harvesting (Gill and Tuteja 2010).

Hypoxic conditions and specially reoxygenation of the cells, promote a redox imbalance of the mitochondria and chloroplast components leading to over reduction of electron carriers and to electron leaking (Murphy 2009) allowing them to react with oxygen to produce reactive oxygen species (ROS) (Halliwell 2006). In order to cope with oxidative damage, plants possess an efficient antioxidative defence system, composed of both enzymatic and non-enzymatic components (Yang et al. 2011; Alhdad et al. 2013).

Among enzymatic system, superoxide dismutase (SOD) is the key enzyme in the antioxidative defence system reported to play an important role of scavenging superoxide radical ($O_2^{\bullet -}$) anion into hydrogen peroxide (H_2O_2), under hypoxic and recovery conditions (Garnczarska 2005; Sairam et al. 2008; Kumutha et al. 2009; Simova-Stoilova et al. 2012). Further, H_2O_2 is breakdown into water and dioxygen by catalase (CAT), guayacol peroxidase (GPOD) or ascorbate peroxidase (APX) (Blokhina and Fagerstedt 2010a; Gill and Tuteja 2010), with different responses upon hypoxia and recovery (Garnczarska 2005; Shi et al. 2008). Among the non-enzymatic antioxidants, which are generally small molecules, ascorbate (AsA) plays a key role in the destruction of H_2O_2, together with glutathione and glutathione reductase (GR) via ascorbate-glutathione cycle (Noctor et al. 1998) and exist mostly in its reduced form in leaves and roots (Smirnoff 2000).

Another class of enzyme with potential antioxidant properties is glutathione S-transferase (GST), a well-known enzyme acting in the detoxification of herbicides that also can act as antioxidant by tagging oxidative degradation products as fatty acids and nucleic acids, for removal or by acting as a peroxidase to directly scavenge peroxides and remove lipid peroxidation (Dalton et al. 2009).

Recently, many studies have been reported that exogenous supply of nitrate plays an important role in several plant species under hypoxia, such as soybean (Thomas and Sodek 2005; Oliveira et al. 2013 a,b), rice (Reggiani et al. 1985), tobacco (Stoimenova et al. 2003) and tomato (Allègre et al. 2004) by improving the redox state and adenylate energy charge (Lanza et al. 2014). Although, the mechanism by which nitrate exerts the beneficial effect during hypoxia are not completely understood (Bailey-Serres and Voesenek 2008). It has been attributed to NO production via nitrate reductase catalysis in the cytosol, acting in the recycle of NAD^+ from NADH (van Dongen and Licausi 2015) or via reduction of nitrite via cytochrome *c* oxidase (COX), linked to membrane proton translocation within mitochondria (Gupta et al. 2005; Wulff et al. 2009; Gupta et al. 2011; Gupta and Igamberdiev 2011),

resulting in NADH oxidation and ATP production (Stoimenova et al. 2007; Horchani et al. 2011).

Although several studies have been focused on the beneficial effects of nitrate (NO_3^-) under waterlogged conditions, including soybean (Thomas and Sodek 2005; Horchani et al. 2011; Lanza et al. 2014), there is no information regarding its effects on antioxidant status of waterlogged root system of non-nodulated and nodulated soybean plants which, cultivated in the absence of mineral N, are naturally free of endogenous nitrate and therefore not metabolically adapted to its presence. Therefore, in this work, we verified whether nitrate exerts beneficial effects on the antioxidant system of soybean plants, growing on presence (non-nodulated) and absence (nodulated) of nitrate.

Materials and methods

Plant material and growth conditions

The study was carried out with two soybean genotypes (*Glycine max* L. Merril) "Fundacep 53 RR" and "BRS Macota", respectively tolerant and sensitive to hypoxia (Borella et al. 2014). An experiment was done with two groups, nodulated and non-nodulated plants grown in greenhouse under natural light and temperature conditions. Plants were cultivated in 3 L pots (three plants per pot) in vermiculite and supplied twice a week with 250 mL of N (NO_3^-) nutrient solution (for non-nodulated plants) or N-free nutrient solution (for nodulated), as described previously by Lima and Sodek (2003). Nodulated plants were inoculated when the cotyledons were fully open by applying 2.5 mL of liquid medium containing 10^9 cells mL^{-1} of *Bradyrhizobium elkanii* strain SEMIA 587 (FEPAGRO), around the stem of each plant on two occasions at 3-d intervals. Treatments were initiated with plants at stage R2 [for stage definitions, see Fehr et al. (1971); R2 = flowering (early reproductive stage)].

For the hydroponic treatment, plants were removed from pots and the root system carefully washed in tap water to remove the vermiculite before transferring to 3 L pots (3 plants per pot) containing N-free nutrient solution (for nodulated plants) or N (5 mM NO_3^-) nutrient solution, both at one-third of normal strength. The whole root system was kept submersed in the nutrient solution. The root system was subjected to hypoxia by flushing N_2 gas for 24 and 72 h. Oxygen concentration in the solution was monitored with an oxygen meter (Handylab OX1). For recovery, after 72 h of hypoxia, plants were returned back to 3 L pots containing vermiculite as substrate under normoxic conditions per 24 and 72 h. Plants

maintained continuously in vermiculite were used as control. At the harvest, four biological replicates of roots and leaves were taken up for each treatment and kept frozen (- 80ºC) until analysis.

Enzymatic activity assays

For the measurement of enzyme activities, leaves and roots (± 0.2 g) were ground using liquid N_2 in porcelain mortars, containing 5% (w:v) polyvinylpolypyrrolidone (PVPP) and homogenized in 1.8 mL of 100 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM sodium ascorbate. The homogenate was centrifuged at 12000 g for 20 min and the supernatant obtained was used as crude enzyme extract. All steps in the preparation of the enzyme extract were carried out at 4°C. An aliquot of the extract was used to determine protein content by Bradford (1976) utilizing bovine serum albumin as standard.

SOD activity (EC 1.15.1.1) was assayed as described by Giannopolitis and Ries (1977) by monitoring the inhibition of the nitroblue-tetrazolium (NBT) coloration at 560 nm in a reaction containing 50 mM potassium phosphate buffer, pH 7.8, 14 mM methionine, 0.1 µM EDTA, 75 µM NBT and 2 µM riboflavin. One unit of SOD activity was defined as the amount of enzyme that produces 50% inhibition of the photochemical reduction of NBT. CAT activity (EC 1.11.1.6) was determined by using the method described by Azevedo et al. (2006). Assay mixture consisted of 100 mM potassium phosphate buffer, pH 7.0, with 12.5 mM hydrogen peroxide and crude enzyme extract. CAT activity was measured as decline in absorbance at 240 nm ($\varepsilon = 39.4 \times 10^3\ M^{-1}\ cm^{-1}$). APX activity (EC 1.11.1.11) was determined according to the method described by Nakano and Asada (1981). The reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, 0.5 mM sodium ascorbate, 0.1 mM hydrogen peroxide and an aliquot of enzyme. The reaction was started by the addition of hydrogen peroxide and the rate of ascorbate oxidation was monitored at 290 nm ($\varepsilon = 2.80 \times 10^3\ M^{-1}\ cm^{-1}$). GR activity (EC 1.6.4.2) was assayed according to Cakmak et al. (1993) by following the decrease in absorbance at 340 nm due to NADPH oxidation ($\varepsilon = 6.2 \times 10^3\ M^{-1}\ cm^{-1}$). The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.8, 1 mM oxidized glutathione (GSSG), 75 µM NADPH and an enzyme aliquot. GPOD activity (EC 1.11.1.7) were assayed following the method described by Urbanek et al. (1991) by monitoring the tetraguayacol production by reduction of hydrogen peroxide at 470 nm (ε

=26.6 x 10^3 M^{-1} cm^{-1}). The reaction consisted of 100 mM potassium phosphate buffer, pH 7.0, 0.1 µM EDTA, 5 mM guayacol and 15 mM hydrogen peroxide.

GST activity (EC 2.5.1.13) was performed as described by Dalton et al. (2009). The extraction buffer consisted of 250 mM Tris-HCl, pH 7.8, 1.0 mM EDTA, 5 mM β-mercaptoethanol, and 0.5% Triton X-100. After centrifuging at 13000g for 10 min, the supernatant was assayed for GST activity with 2,4-dinitrochlorobenzene (CDNB) as substrate. Activity with CDNB was measured in a reaction containing crude enzymatic extract, 1.0 mM reduced glutathione (GSH), 1.0 mM CDNB, and 0.1 M potassium phosphate buffer, pH 7.5. The protein extract was added last, and the absorbance was monitored at 340 nm (ε =6.2 x 10^3 M^{-1} cm^{-1}).

Ascorbate content

The contents of ascorbate reduced (AsA) and total ascorbate [(AsA + oxidized ascorbato (DHA)] were quantified as described by Arakawa et al. (1981) with some modifications. Samples from roots or leaves (0.2 g) were ground in 5% trichloroacetic acid (TCA), homogenized and centrifuged at 10000 g for 15 min at 4ºC. Total ascorbate from supernatant was determined after reduction of DHA by dithiothreitol (DTT). The reaction medium consisted of 5% TCA, 0.06% DTT and 0.2 M sodium phosphate buffer, pH 7.0. After incubation at room temperature for 10 min, 0.24% N-ethylmaleimide was added and the pH of each tube adjusted to between 1 and 2 with 20% TCA. After that, were added in a final concentration 4% phosphoric acid (H_3PO_4), 0.5% bathophenanthroline and 0.03% ferric chloride ($FeCl_3$) and incubated at 30ºC for 90 min. The absorbance was read at 534 nm. The ascorbate was determined as described above, but replacing the DTT by absolute ethanol in equal volume. The values for DHA were obtained by the difference between the values of total ascorbate and reduced ascorbate. The ascorbate redox state was calculated as [(AsA)/(AsA + DHA)] x 100 and expressed as percent (Bonifacio et al. 2011).

$O_2^{\bullet-}$ content

The assay of $O_2^{\bullet-}$ generation rate was determined according to Li et al. (2010). The tissues (0.2 g) were ground in 65 mM phosphate buffer, pH 7.8, and centrifuged at 5000 g for 10 min. The supernatant was mixed with 65 mM phosphate buffer, pH 7.8, and 10 mM

hydroxylamine hydrochloride, and placed at 25ºC for 20 min. Then 17 mM sulfanilamide and 7 mM α-anaphthylamine in a final concentration, were added to the mixture. The absorbance of the solution at 530 nm was measured after incubation for 20 min at 25ºC. A standard curve with nitrite dioxide radical (NO_2^-) was used to calculate the $O_2^{\bullet -}$ generation rate.

H_2O_2 content

Hydrogen peroxide levels were determined according to Velikova et al. (2000). The tissues (0.2 g) were ground in 0.1% (w:v) trichloroacetic acid (TCA). The homogenate was centrifuged (12000 g, 4ºC, 20 min) and the supernatant was added to 10 mM potassium phosphate buffer, pH 7.0 and 1 M potassium iodide. The absorbance of the reaction was measured at 390 nm. The content of H_2O_2 was given on a standard curve prepared with known concentrations of H_2O_2.

Lipid peroxidation measurement

For the measurement of lipid peroxidation the thiobarbituric acid (TBA) test, which determines malondialdehyde (MDA) as an end product of lipid peroxidation, was used. The material (0.1 g) was homogenized in 0.1% (w:v) TCA solution. The homogenate was centrifuged (12000 g, 4ºC, 20 min) and the supernatant was added to 0.5% (w:v) TBA in 10% TCA solution. The mixture was incubated in boiling water (90ºC) for 20 min, and the reaction stopped by placing the reaction tubes in an ice bath for 10 min. Then the samples were centrifuged at 10000g for 5 min, and the absorbance was read at 535 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient ($\varepsilon = 155 \times 10^3\ M^{-1}\ cm^{-1}$).

Statistical analysis

Each treatment consisted of four replicates, where each replicate consisted of one pot containing three plants (material pooled), in a fully randomized design. The data were analysed by one-way analysis of variance (ANOVA). When *F* was significant the treatments means for each genotype or the N assimilation for each treatment were compared by Tukey's

test ($p \leq 0.05$). Statistical analyses were performed using the SAS 8.0 statistical software program (SAS Institute Inc. Cary, NC, USA).

Results

The hypoxic system was set up by flushing N_2 gas into the pots containing the whole root system in the nutrient solution at one-third of normal strength. Initially, the oxygen concentration into the solution of both, nodulated and non-nodulated, was about 6.5 mg L^{-1} (normoxia). The concentration decreased rapidly to 0.5 mg L^{-1} within 5 h and reaching 0.25 mg L^{-1} in 24 h until the end of the experiment (72 h) (data not shown), similar to those reported by Borella et al. (2014).

Antioxidant enzymatic activity

The induction of the plant's antioxidant enzymatic system to hypoxia and recovery is shown in both, roots (Fig 1 and 3) and leaves (Fig. 2 and 4), of nodulated (nitrate free) and non-nodulated (nitrate-supplied) soybean plants of two soybean genotypes, Fundacep 53 RR and BRS Macota. The activity of the enzymes increased significantly in roots during hypoxia and recovery in non-nodulated plants. SOD and CAT increased in roots of both waterlogged non-nodulated soybean genotypes at 72 h, and kept higher than control during the recovery with a strong effect in Fundacep 53 RR. In nodulated soybean plants an increase in the SOD activity was observed at 24 h of hypoxia in both genotypes and remained until the end of the experiment in BRS Macota. In Fundacep 53 RR the activity decreased with the recovery. CAT activity did not differ from the control during the entire experiment in both genotypes. Plants supplied with nitrate increased SOD and CAT activities about 3-fold higher compared with nodulated plants (Fig. 1 A and B).

APX activity only increased upon return to normoxia in nitrate-supplied plants while did not differ in nodulated plants in both genotypes (Fig. 1 C and D). Interestingly, APX activity was responsive upon recovery with a higher increase in the activity in nitrate-supplied plants and a faster increase in Fundacep 53 RR than BRS Macota (Fig. 1).

In leaves, the enzyme activities were differently from roots under normoxic conditions. Despite of increased activity of SOD and APX with the recovery in plants supplied with nitrate (Fig. 2), these enzymes did not change the activity in nodulated plants, except the

activity of SOD in BRS Macota at 72 h of hypoxia (Fig. 2 B). In addition, SOD and APX were higher in nodulated plants than in plants supplied with nitrate (non-nodulated) in comparison their activities in roots (Fig. 1). On the other hand, CAT activity appears to be not responsive in leaves (Fig. 2 E and F) as it is in roots.

In addition to SOD, APX and CAT (Fig. 1 and 2), the activity of GR and GPOD increased in roots in a similar way of CAT with 72 h of hypoxia, though they were higher during the recovery, in nitrate-supplied plants (Fig. 3), with a remarkable increase of GR in Fundacep 53 RR. GR activity did not increase in nodulated plants. In leaves, GR was more active during hypoxia with increased activity at 72 h in leaves of both genotypes in nitrate-supplied plants while in nodulated plants the activities were similar to control (Fig. 4 A and B). In contrast, GPOD was found the most active peroxidase in roots under hypoxia (Fig. 3 C and D), whilst no activity of this enzyme was detected in leaves.

GST activity changed significantly in roots during hypoxia in non-nodulated plants, decreasing in Fundacep 53 RR and increasing with the reoxigenation. In BRS Macota, GST activity increased during hypoxia and kept the high levels during recovery (Fig. 3 E and F). In leaves, GST activity increased markedly during hypoxia in both genotypes and kept higher levels of activity during recovery (Fig. 4 C and D). In nodulated plants GST was more active during hypoxia in roots and leaves (Fig. 4 C and D) of two genotypes.

Ascorbate redox state

Ascorbate plays essential role in abiotic stress in plants. Changes in reduced ascorbate was higher in nitrate-supplied plants though an increased content occurred during recovery in nodulated and non-nodulated plants, compared to the content during hypoxia in both, roots (Fig. 5) and leaves (Fig. 6) of Fundacep 53 and BRS Macota. On the other hand, increased ascorbate redox state was higher during recovery only in non-nodulated plants in roots and leaves, and these responses were higher in BRS Macota genotype.

Oxidative damage

Increases in the steady state level of the relatively stable reactive oxygen species and membrane lipid peroxidation products are considered to reflect oxidative stress. Superoxide, hydrogen peroxide and lipid peroxidation in roots and leaves are shown in Fig. 7 and 8, respectively. In roots of nitrate-supplied plants with 24 h of hypoxia the content of superoxide

was kept similar to the control and further reduced with 72 h of hypoxia in both genotypes, whereas with the recovery in Fundacep 53 RR the content kept below to the control levels (Fig. 7 A and B), reflecting the activity of SOD in roots (Fig. 1 A and B). In nodulated plants the production of superoxide decreased during hypoxia and increased with the return to normoxic conditions (Fig. 7 A and B).

Hydrogen peroxide content was lower even under hypoxia and recovery in roots of plants supplied with nitrate than nodulated plants, which showed a slightly decrease in its production (Fig. 7 C and D) due to the activity of the enzymes responsive for its scavenge. A decreased level of lipid peroxidation was exhibited in roots of both genotypes upon hypoxia and recovery. However, the modulation of the levels was higher in plants supplied with nitrate, which did not increase the levels to the control after hypoxia as in roots of nodulated plants in both genotypes (Fig. E and F).

In leaves, differently from roots, an increase in superoxide production was exhibited by nitrate-supplied plants during hypoxia and kept higher levels than control even at 72h of recovery. In nodulated plants, the content did not change in Fundacep 53 RR and increased in BRS Macota during hypoxia and decreasing to the control level at 72h of recovery (Fig. 8 A and B). Hydrogen peroxide production were lower than control at 24h of hypoxia and increased later, kept to the control levels during recovery in nodulated plants. In non-nodulated plants there was no significative change in hydrogen peroxide levels (Fig. C and D). Lipid peroxidation did not alter significantly during hypoxia and post-hypoxia treatments in Fundacep 53 RR nodulated plants and increased at 72 h of hypoxia in BRS Macota, decreasing to the control levels during recovery. In non-nodulated plants, there was no significative change in lipid peroxidation during hypoxia in comparison to normoxia for both genotypes. During post-hypoxia treatments there was a decrease in lipid peroxidation in Fundacep 53 RR and no alteration in BRS Macota (Fig. 8 E and F).

Discussion

In this work we describe the involvement of nitrate in alleviating the effects of oxidative damage caused by ROS via induction of antioxidant enzymatic and non-enzymatic during and after hypoxia in leaves and mainly in roots of non-nodulated plants (plants assimilating nitrate) in comparison to nodulated plants (plants assimilating ammonium, via N_2 fixation) of two soybean genotypes Fundacep 53 RR and BRS Macota.

Since the early study of Arnon (1937), nitrate has been investigated and find out to exert beneficial effects in plants under oxygen deficiency (Allegre et al. 2004; Horchani et al. 2010; Horchani et al. 2011). However, these studies have been concentrated on primary carbon and nitrogen metabolism (Thomas and Sodek 2005; Horchani et al. 2010; Oliveira et al. 2013a,b; Oliveira and Sodek 2013; Lanza et al. 2014). In agreement with these previous works regarding nitrate effects, a similar pattern was also observed in this work in roots of non-nodulated plants with the induction of the enzymes SOD, APX, CAT, GR and GPOD (Fig. 1 and 3) and non-enzymatic antioxidant (Fig. 5), as well as their efficiency in scavenge the production of ROS (Fig. 7) in response to hypoxia and recovery. These results clearly demonstrated differences between the two forms of nitrogen assimilation, nitrate (non-nodulated plants) and ammonium (nodulated plants) regarding induction of antioxidant metabolism.

In leaves, despite of the slight induction of antioxidant system, an increase of enzymatic and non-enzymatic antioxidants were shown (Fig. 2, 4 and 6). These results might be due to the short period of hypoxia (3 days) that plants were submitted and also that leaves were kept under normoxic conditions, which can alleviate the effects of the oxygen deprivation somehow in comparison with roots, which are directly affected.

In non-nodulated plants an increased induction of SOD was shown in roots under hypoxia. During recovery its induction was much stronger, at least in Fundacep 53 RR, while in nodulated plants SOD activity appears to be more induced under hypoxic conditions (Fig. 1 A and B). These results are in agreement with the reduction of $O_2^{\bullet-}$ anion production by SOD activity (Fig. 7 A and B). Furthermore, in non-nodulated roots of Fundacep 53 RR, $O_2^{\bullet-}$ production did not reach levels of the control under recovery (Fig. 1 A) due to its correspondent high SOD activity (Fig. 1 A).

As reported, SOD constitutes the first line of defence against ROS, playing an important role in the detoxification of $O_2^{\bullet-}$ into H_2O_2 (Blokhina and Fagerstedt 2010a,b; Gill and Tuteja 2010). Although, the production of ROS has been shown to increase in several plant species under hypoxia (Bai et al. 2010; Bansal and Srivastava 2012; Simova-Stoilova et al. 2012), it also has been correlated to the time of the hypoxic treatment (Blokhina and Fagerstedt, 2010b). On the other hand, it has been reported that under hypoxia conditions ROS production decline due to oxygen deprivation and it is even abolished in anoxic conditions (Sairam et al. 2011). Moreover, a differential SOD response to oxygen deprivation stress (anoxia and hypoxia) on different plants has been always contradictorily described, depending

of the experimental set-up or prolonged reoxygenation (Blokhina et al. 2003). The decline in $O_2^{\bullet-}$ and H_2O_2 production under hypoxia was attributed to a shift from aerobic respiration to fermentation with the blockage of the mitochondrial site of ROS production (Sairam et al. 2011).

In nitrate-supplied plants (soybean), fermentation has been reported to be modulated by NO production leading to a decreased production of lactate and ethanol (Oliveira et al. 2013a,b), once NO acts as an alternative pathway to recycle NAD^+ from NADH under low-oxygen conditions via futile nitric oxide (NO) cycle, where nitrite reduction by nitrate reductase lead to NO production in the cytosol (Limami et al. 2014; van Dongen and Licausi 2015). Another pathway is the reduction of nitrite via cytochrome *c* oxidase (COX), linked to membrane proton translocation (Gupta et al. 2005; Wulff et al. 2009; Gupta et al. 2011; Gupta and Igamberdiev 2011; Oliveira et al. 2013b). NO is then oxidized to nitrate again by class-1 nonsymbiotic hemoglobin (Igamberdiev and Hill 2004), and it may have an involvement in the induction of antioxidant system observed in roots of nitrate-supplied plants.

NO has emerged as an important free radical signal in plants (Neill et al. 2008). We suggest that NO may have some effect in the modulation of antioxidant system by induction of SOD (Fig. 1 A and B) in roots of non-nodulated plants. ROS act in oxidative damage to membrane cells (Blokhina and Fagerstedt 2010b; Gill and Tuteja 2010), with deleterious consequences and signalling roles in biological systems (Blokhina and Fagerstedt 2010a). Among the consequences are damage to proteins, lipids, carbohydrates and DNA which ultimately results in cell death (Gill and Tuteja 2010). However, to confirm the involvement of NO in the antioxidant modulation further investigations are needed.

Although the NO production is enhanced upon hypoxia (Gupta et al. 2011), the increased activity of the antioxidant enzymes (Fig. 1 and 3) under recovery conditions might be the extensive reflect of hypoxia and the effects of the reoxygenation which is well reported as being responsive for oxidative burst in the cells, thus leading to enzyme induction to counteract possible oxidative damage (Garnczarska 2005; Sairam et al. 2008; Kumutha et al. 2009; Simova-Stoilova et al. 2012)

In addition to SOD, CAT (Fig. 1 E and F), GR (Fig. 3 A and B) and GPOD (Fig. 3 C and D) were shown to have important role in the detoxification of H_2O_2 in roots of non-nodulated plants in both genotypes under hypoxia and recovery (Fig. 7 C and D). APX, another enzyme acting in the scavenge of H_2O_2 was responsive upon return to normoxic conditions in roots (Fig 1 C and D) of non-nodulated plants, in agreement with gene

expression and activity of APX in seedling of soybean which were found to be responsive only after hypoxic stress (SHI et al., 2008). In nodulated plants only GPOD showed importance in the detoxification of H_2O_2, either under hypoxia and recovery (Fig. 3 C and D). Blokhina et al. (2003) arose with the hypothesis that CAT acts earlier in response to hydrogen peroxide production compared to other enzymes, explaining, in part its high activity in roots.

APX and GR are reported as being responsible for scavenge H_2O_2 (Blokhina and Fagerstedt 2010a,b; Gill and Tuteja 2010) together with non-enzymatic antioxidants via ascorbate-glutathione cycle (Bonifacio et al. 2014), where GR and GSH are used to reduce back AsA, oxidized by APX (Blokhina and Fagerstedt 2010b). The redox states of GSH and AsA in roots of wheat were reported to be directly dependent on oxygen concentration and reflected oxidative burst upon re-aeration (Biemelt et al. 1998). Although, ROS production did not increase in roots (Fig. 7) upon recovery, the increased ascorbate redox state and decreased AsA might be resulted from APX (Fig. 1 and 2) and GR activity (Fig. 3 and 4). On the other hand, CAT was not responsive in leaves (Fig 2 E and F) as in roots, while APX and GR increased mainly in response to recovery which may be explained by the fluctuation of NAD(P)H/$NAD(P)^+$ ratio under hypoxia (Stoimenova et al. 2007) to keep the ascorbate-glutathione cycle operating properly, whereas CAT itself does not need NAD(P)H to breakdown H_2O_2 (Blokhina and Fagerstedt 2010b).

On the other hand, dehydroascorbate (DHA) was shown to be taken up by mitochondria, which suggests that the mitochondrial respiratory electron chain of plant cells plays an important role not only in the synthesis of ascorbate but also in the regeneration of ascorbate from its oxidized form, DHA (Blokhina and Fagerstedt 2010b; Gill and Tuteja 2010). Interestingly, DHA was also been shown to participate efficiently in the scavenge of NO (Kytzia et al. 2006).

In addition to efficient enzymatic system operating to scavenge $O_2^{\bullet -}$ and H_2O_2, avoiding lipid peroxidation in roots (Fig. 7 E and F) and leaves (Fig. 8 E and F), the enzyme GST might have an important role by tagging oxidative degradation products (fatty acids and nucleic acids) for removal or by acting as a peroxidase to directly scavenge peroxides and remove lipid peroxidation products (Dalton et al. 2009). ROS are dangerous because of their ability to initiate a chain reaction on polyunsaturated fatty acids that leads to lipid peroxidation (BAI et al., 2010). Free fatty acids (FFAs) are recognized as powerful uncoupling agents activating mitochondrial UCPs and leading to a severe membrane damage and further cell death (Blokhina and Fagerstedt 2010b).

Efficient antioxidant system as shown in roots of both genotypes (Fig. 1 and 3) had an important role against ROS (Fig. 7) avoiding them to affect uncoupling protein properties of the mitochondria as reported in wheat (Grabel'nych et al. 2009) and to affect membrane fluidity (Schönfeld and Wojtczak 2008), once in non-nodulated plants, nitrate exert important role on mitochondrial electron transport chain leading to ATP synthesis under hypoxia (Horchani et al. 2011) via oxidation of NADH and NADPH (Stoimenova et al. 2007).

Waterlogging has been also reported to induce stomatal closure, decrease in leaf chlorophyll and carotenoids content, production of ethylene and disruption of the translocation of photosynthates (Blokhina and Fagerstedt 2010a;b). It may have influenced in part the high production of $O_2^{\bullet-}$ in leaves (Fig. 8 A and B). Furthermore, the increase in SOD activity in leaves (Fig. 2 A and B) might be reflect from $O_2^{\bullet-}$ production, once NO was not reported to be produced from nitrite reduction in leaves under hypoxic conditions (Gupta et al. 2005), although nitrate is transported through xylem sap from roots to shoot in soybean plants (Oliveira et al. 2013a; Lanza et al. 2014) and the lack ability of leaf mitochondria to produce NO might somehow be related to photosynthesis (Gupta et al. 2005).

Despite of the genotypes Fundacep 53 RR and BRS Macota have been reported as tolerant and sensitive under hypoxia, respectively (Borella et al. 2014) the antioxidative metabolism here studied was not found to be correlated with tolerance mechanism that differentiate genotypes, might be due to the short-term of flooding of the root system (Wang et al. 2009). However, as well reported tolerant species increase the activity of antioxidant enzymes to counteract the oxidative effects (Kumutha et al. 2009; Sairam et al. 2009; Simova-Stoilova et al. 2012) which is in accordance with our findings and others well reported once nitrate exerts beneficial effects on soybean plant by inducing antioxidant enzymatic and non-enzymatic compounds that can lead to a prolonged tolerance in comparison to non-nitrate-supplied plants.

Conclusions

Our data reveal that nitrate exerts beneficial effects in soybean plants under hypoxic conditions and consequent recovery by inducing the antioxidant system manly in roots, to cope possible oxidative damage caused by ROS production. It was also demonstrated that in soybean plants enzymatic antioxidant system is much more responsive during recovery from hypoxia stress than during the period of oxygen privation. Furthermore, our findings have

arisen a possible influence of NO in modulating the antioxidant system which deserves further investigations.

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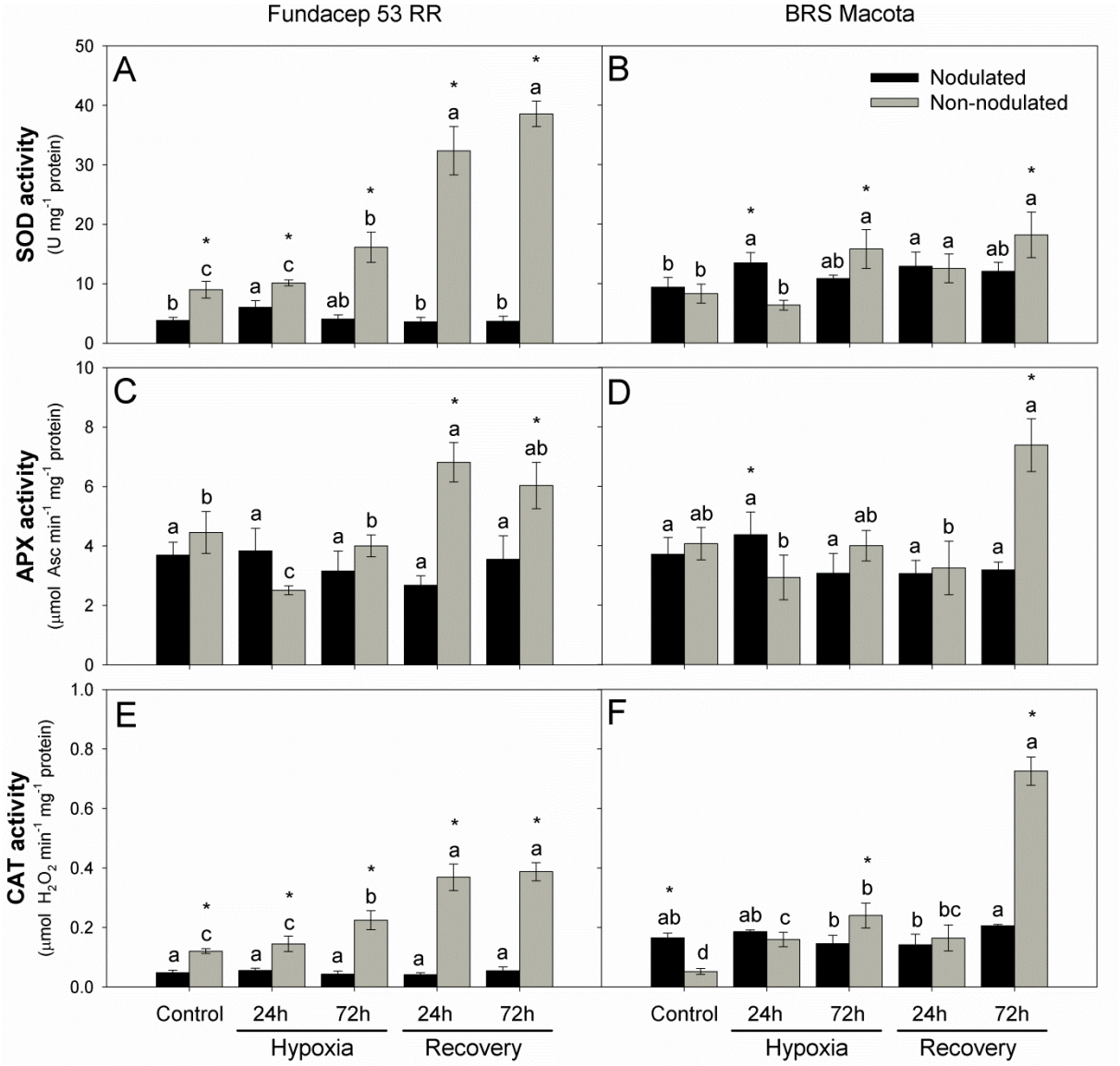

Fig. 1 – Superoxide dismutase (SOD – A and B), ascorbate peroxidase (APX – C and D) and catalase (CAT – E and F) activity in roots of nodulated and non-nodulated soybean of two genotypes (Fundacep 53 RR and BRS Macota) under conditions of hypoxia and recovery. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for nodulated or non-nodulated, separately. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between nodulated and non-nodulated for each treatment. Values represent the mean ± SD ($n = 4$).

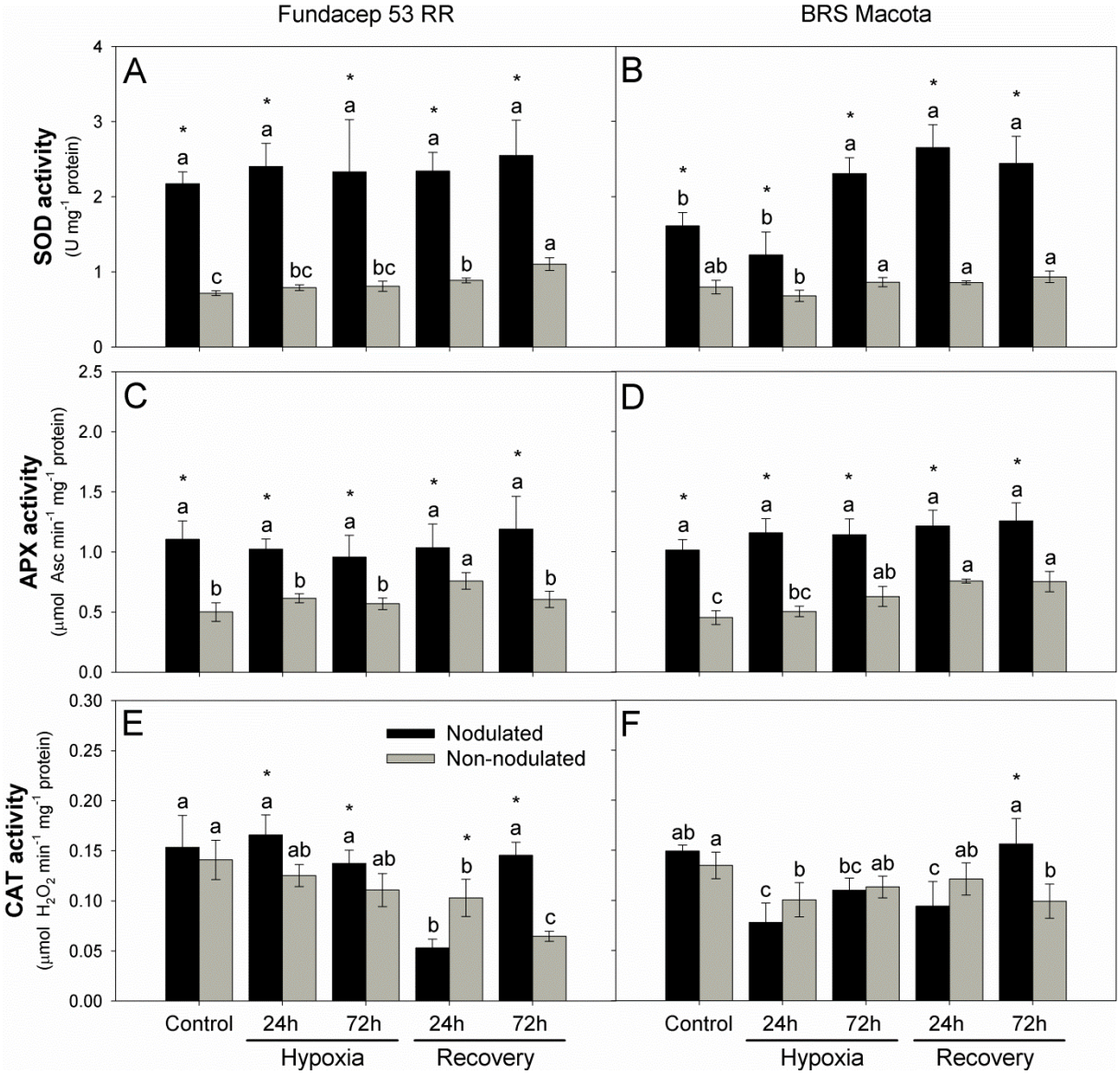

Fig. 2 – Superoxide dismutase (SOD – A and B), ascorbate peroxidase (APX – C and D) and catalase (CAT – E and F) activity in leaves of nodulated and non-nodulated soybean of two genotypes (Fundacep 53 RR and BRS Macota) under conditions of hypoxia and recovery. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for nodulated or non-nodulated, separately. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between nodulated and non-nodulated for each treatment. Values represent the mean ± SD ($n = 4$).

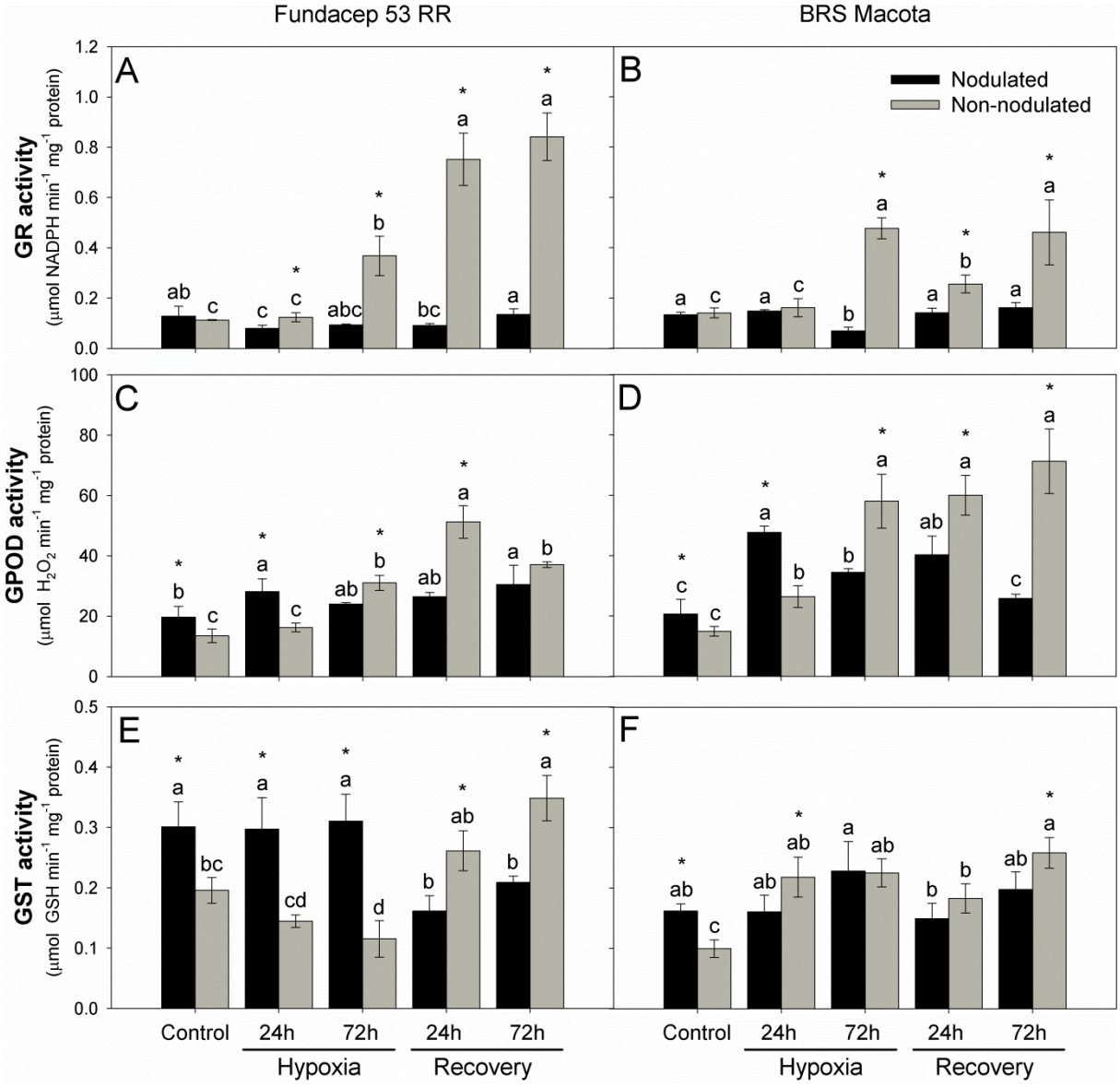

Fig. 3 – Glutathione reductase (GR – A and B), guayacol peroxidase (GPOD – C and D) and glutathione S-transferase (GST – E and F) activity in roots of nodulated and non-nodulated soybean of two genotypes (Fundacep 53 RR and BRS Macota) under conditions of hypoxia and recovery. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for nodulated or non-nodulated, separately. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between nodulated and non-nodulated for each treatment. Values represent the mean ± SD ($n = 4$).

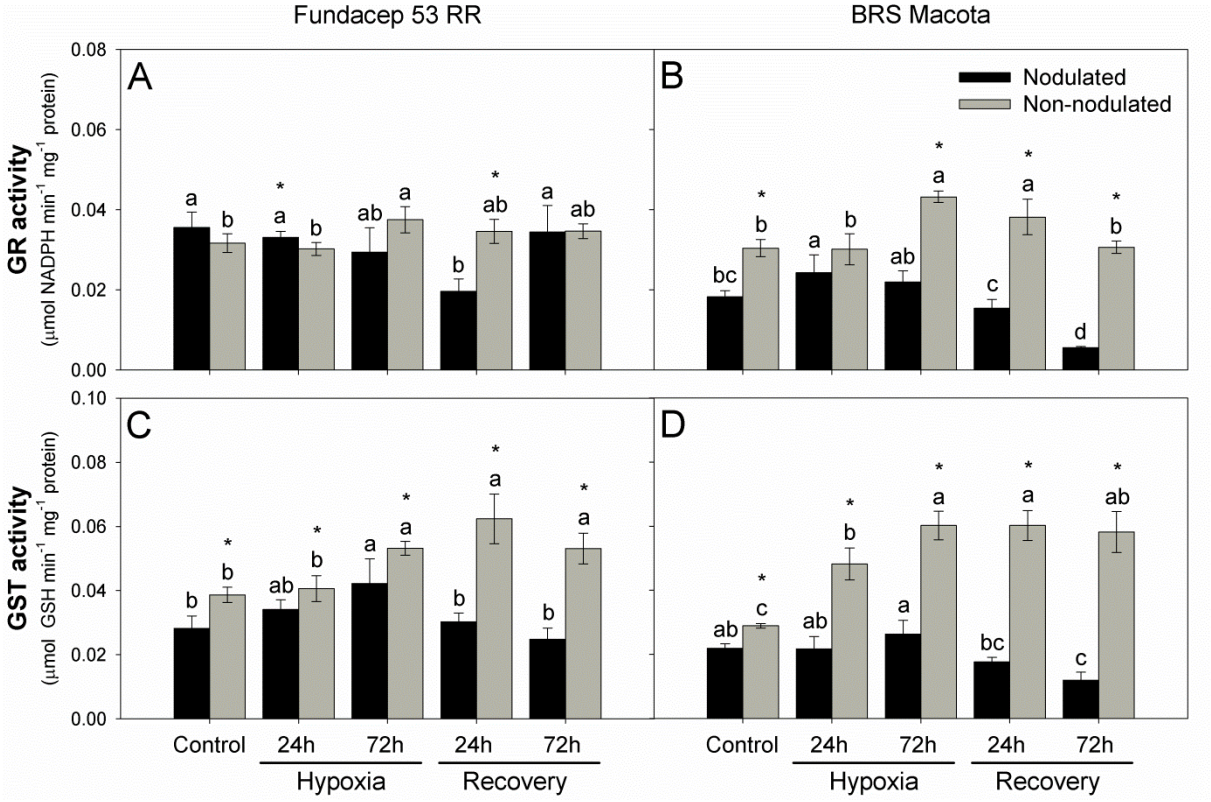

Fig. 4 – Glutathione reductase (GR – A and B), glutathione S-transferase (GST – C and D) activity in leaves of nodulated and non-nodulated soybean of two genotypes (Fundacep 53 RR and BRS Macota) under conditions of hypoxia and recovery. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for nodulated or non-nodulated, separately. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between nodulated and non-nodulated for each treatment. Values represent the mean ± SD ($n = 4$).

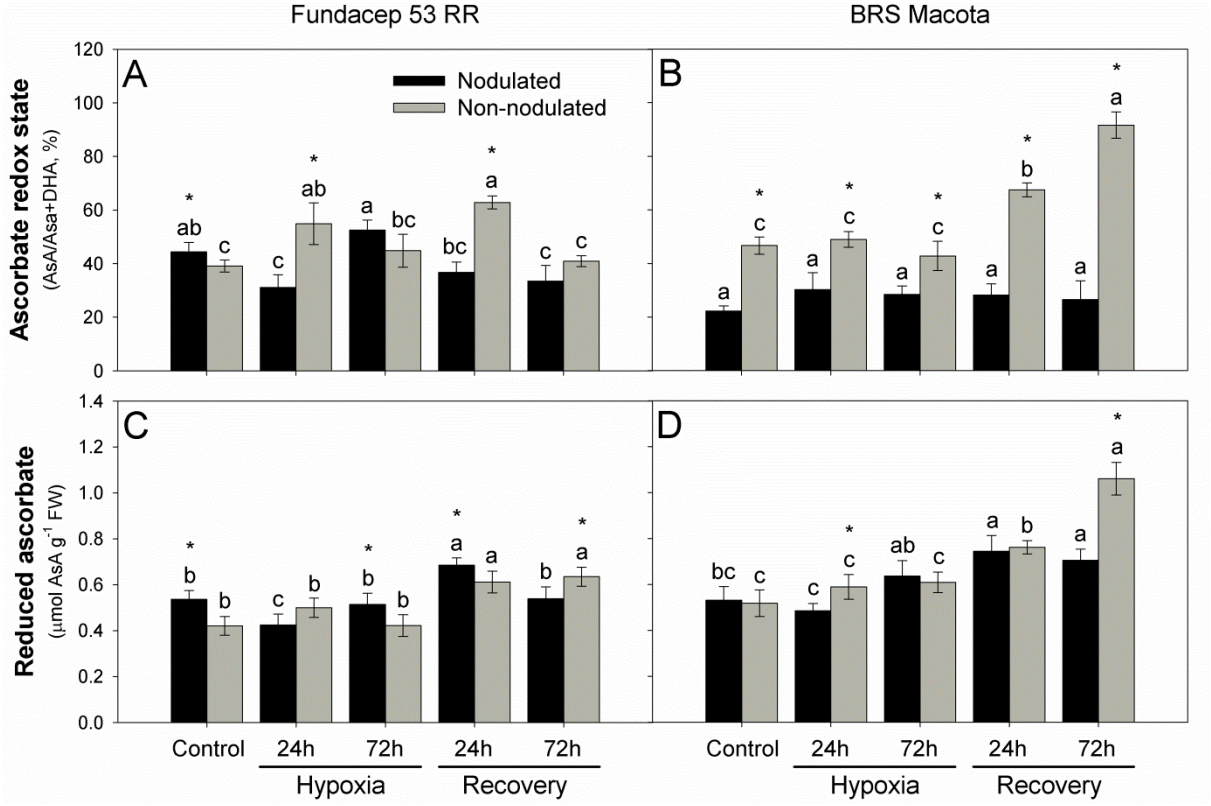

Fig. 5 – Ascorbate redox state (A and B) and ascorbate content (AsA – C and D) in roots of nodulated and non-nodulated soybean of two genotypes (Fundacep 53 RR and BRS Macota) under conditions of hypoxia and recovery. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for nodulated or non-nodulated, separately. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between nodulated and non-nodulated for each treatment. Values represent the mean ± SD ($n = 4$).

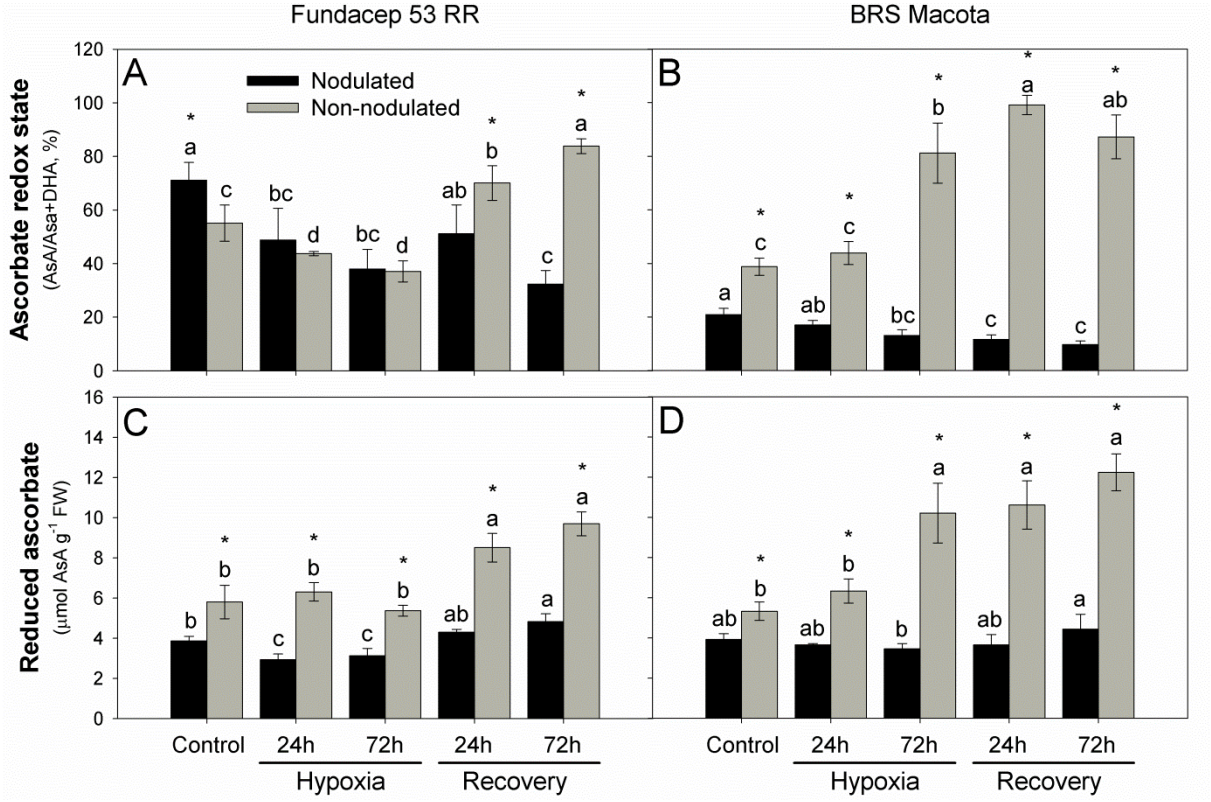

Fig. 6 – Ascorbate redox state (A and B) and ascorbate content (AsA – C and D) in leaves of nodulated and non-nodulated soybean of two genotypes (Fundacep 53 RR and BRS Macota) under conditions of hypoxia and recovery. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for nodulated or non-nodulated, separately. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between nodulated and non-nodulated for each treatment. Values represent the mean ± SD ($n = 4$).

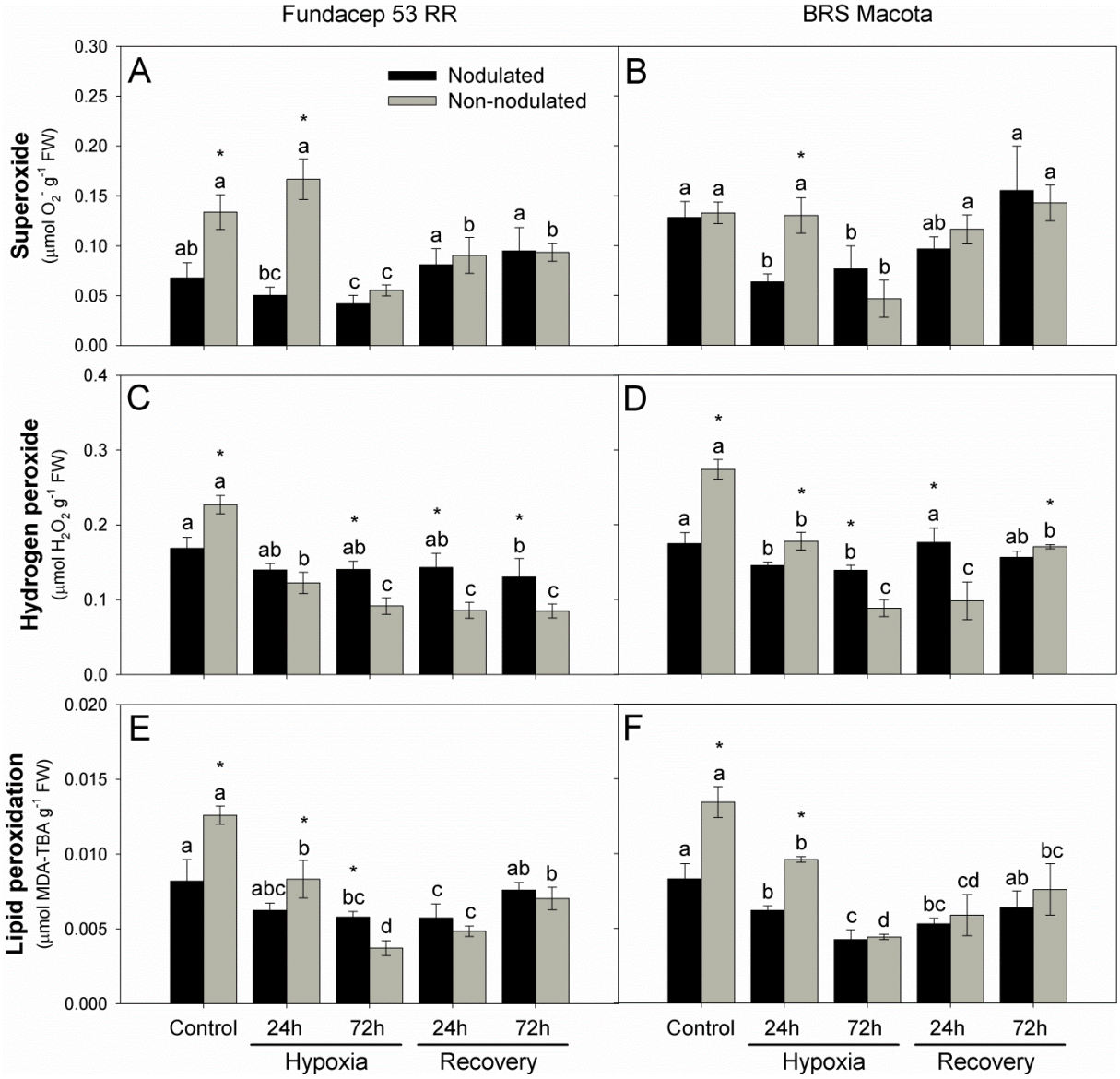

Fig. 7 – Superoxide (A and B), hydrogen peroxide (C and D) content and lipid peroxidation (E and F) in roots of nodulated and non-nodulated soybean of two genotypes (Fundacep 53 RR and BRS Macota) under conditions of hypoxia and recovery. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for nodulated or non-nodulated, separately. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between nodulated and non-nodulated for each treatment. Values represent the mean ± SD ($n = 4$).

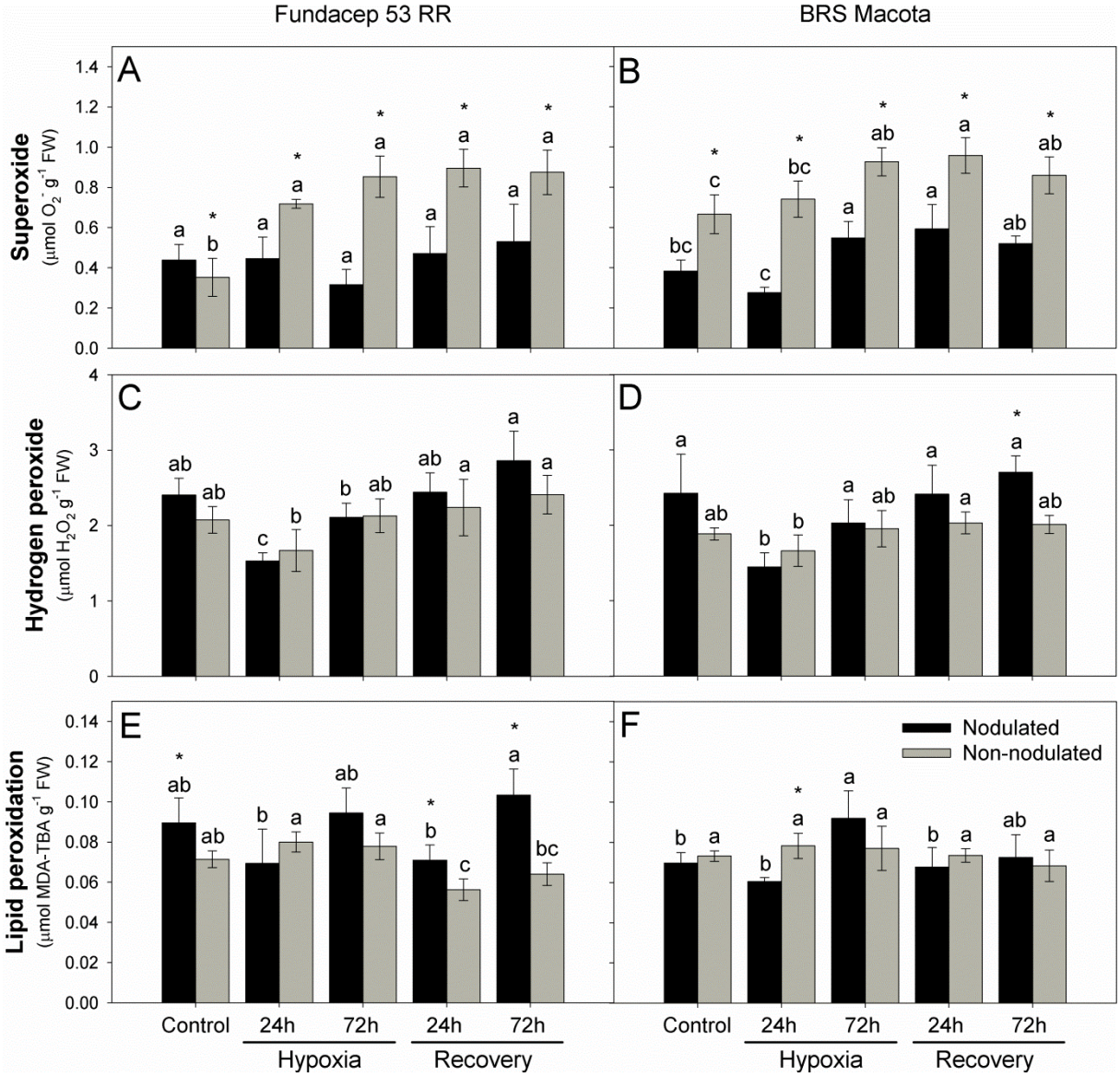

Fig. 8 – Superoxide (A and B), hydrogen peroxide (C and D) content and lipid peroxidation (E and F) in leaves of nodulated and non-nodulated soybean of two genotypes (Fundacep 53 RR and BRS Macota) under conditions of hypoxia and recovery. Means followed by the same letters do not differ by Tukey's test ($p \leq 0.05$) among treatments for nodulated or non-nodulated, separately. Asterisk (*) indicates significant differences by Tukey's test ($p \leq 0.05$) between nodulated and non-nodulated for each treatment. Values represent the mean ± SD ($n = 4$).

Considerações Finais

Em soja, a deficiência de oxigênio no sistema radicular causa o aumento do fluxo glicolítico levando ao acúmulo de piruvato e consequentemente a ativação da fermentação através da produção de lactato para manter a glicólise funcionando, principalmente em raízes, sendo maior o acúmulo desses metabólitos em Fundacep 53 RR comparado à BRS Macota.

Em genótipos de soja, contrastantes à hipóxia, ocorrem alterações nos teores de ácidos orgânicos do ciclo dos ácidos tricarboxílicos, levando ao acúmulo de succinato em Fundacep 53 RR, bem como acúmulo de malato que permitem a manutenção das reações do ciclo sob deficiência de oxigênio sendo o mesmo não observado em BRS Macota.

O metabolismo de aminoácidos faz um *link* entre a glicólise e o TCA através da indução do gene *AlaAT1* e atividade da enzima AlaAT em Fundacep 53 RR, levando ao acúmulo de Ala em raízes e podendo levar a tolerância desse genótipo.

O genótipo Fundacep 53 RR demonstra maior tolerância aos efeitos hipóxicos e pós-hipóxicos em relação à BRS Macota, pois além de apresentar maior eficiência em induzir a expressão e atividade da AlaAT durante a hipóxia, reestabelece mais rápido a atividade e os teores de metabólitos aos níveis pré-hipóxicos com o retorno a normóxia.

Em soja, o nitrato exerce efeito benéfico induzindo a ativação do sistema antioxidante de defesa contra EROs através do aumento da atividade das enzimas SOD, CAT, APX, GR e GPOD em raízes sob condições de hipóxia e pós-hipóxia.

A produção de NO em plantas nutridas com nitrato parece estar envolvida na ativação da SOD sob condições de hipóxia em raízes, enquanto que em folhas a ativação parece estar relacionada à produção direta de EROs influenciados pela hipóxia do sistema radicular, uma vez que folhas são mantidas em condições de normóxia.

O sistema antioxidante é mais responsivo e induzido com o retorno às condições de normóxia do que pelo estresse por deficiência de oxigênio.

O metabolismo do carbono e do nitrogênio via *link* da AlaAT estão envolvidos no mecanismo de tolerância em Fundacep 53 RR comparado à BRS Macota, enquanto que o metabolismo antioxidante parece não exercer influência na tolerância dos genótipos. Por outro lado, a nutrição das plantas com nitrato demonstrou efeito benéfico no sistema antioxidante, o que poderia também exercer efeitos sobre o metabolismo do carbono e do nitrogênio podendo prolongar a tolerância das plantas às condições de deficiência de oxigênio comparado as plantas cultivadas na ausência de nitrato.

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