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CENTRO DE BIOCIÊNCIAS
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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA - RENORBIO

MELQUISEDEC DE SOUSA OLIVEIRA

PERFIL FISIOLÓGICO E PROTEÔMICO DE VARIEDADES DE SORGO SACARINO (*Sorghum bicolor* L. Moench) CONSTRASTANTES EM RESPOSTA À SECA

Recife
2018

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO) da Universidade Federal de Pernambuco, como parte dos requisitos exigidos para obtenção do título de Doutor em Biotecnologia.

Área de Concentração: Biotecnologia em Agropecuária

Orientador: Prof. Dr. Tercilio Calsa Junior

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“Inocência sua achar que o mundo te entenderia, bastava ser sincero” (Se tudo der errado amanhã, 2018).

“Ledo engano!Talvez por isso rogue que meus inimigos sejam fortes e bravos, para que não haja remorso em mim quando lançar cada um ao chão” [PROVÉRBIO APACHE, 2018]

RESUMO

O sorgo sacarino é uma variedade de *Sorghum bicolor* que produz grande concentração de açúcares fermentescíveis nos colmos. Por essa razão, tais plantas representam alternativa para os agricultores que fornecem matéria-prima para a produção de etanol, sobretudo no período de entressafra da cana-de-açúcar. Quando comparado à outras culturas, como o milho e a cana-de-açúcar, o sorgo possui vantagens, como o ciclo de produção mais curto e, principalmente, maior tolerância ao déficit hídrico. Apesar da relevância econômica e biotecnológica, os mecanismos moleculares relacionados à tolerância à seca não estão bem elucidados, sobretudo com base em caracterização fenotípica associada a análise proteômica. Assim, o presente trabalho objetivou determinar as repostas fisiológicas e bioquímicas de diferentes variedades de sorgo sacarino, bem como as principais proteínas relacionadas a resposta à seca, utilizando análise proteômica como ferramenta de análise de expressão gênica. Em experimento de casa de vegetação, plantas das variedades nacionais IPA-SF15, IPA-46742 e EMBRAPA-BR506 foram cultivadas por 55 dias, e então submetidas ao déficit hídrico por suspensão de rega durante 72 h, com posterior reidratação. Foram determinados teor relativo de água, taxa fotossintética, condutância estomática, transpiração, concentração de clorofilas, carotenóides, MDA, prolina, grau Brix^{o}, e atividade de enzimas antioxidantes. A análise proteômica foi realizada comparando-se a variedade fisiologicamente mais sensível e mais tolerante. Em geral, plantas de IPA-SF15 tiveram melhor desempenho em condições de seca, registrando-se maior teor de pigmentos, menor concentração de MDA, maior atividade de enzimas antioxidantes e taxa fotossíntética, em comparação à variedade mais sensível, IPA-46742. A variedade mais tolerante acumulou mais proteínas do ciclo de Calvin (RbcL, PGK), manutenção de fotossistemas (HHL1), síntese de osmólitos (PAO) e metabolitos secundários (AD2). Por outro lado, a variedade mais sensível traduziu mais proteínas de ubiquitinação e inibição de fotomorfogênse, corroborando o fenótipo sensível. Baseado-se no padrão de acúmulo de proteínas, bem como nas respostas fisiológicas, conclui-se que a maior diversidade de mecanismos eficientes empregados por IPA-SF15 representam clara vantagem sob condições de déficit hídrico.

Palavras-chave: Biocombustíveis, Poaceae, gramínea C4, Estresse abiótico

ABSTRACT

Sweet sorghums are varieties of *Sorghum bicolor* with great content of fermentable sugars in the stems. Therefore, these plants are attracting attention of farmers to provide feedstock material for ethanol production, mainly during sugarcane off season. When compared to other biofuel crops, such as maize and sugarcane, sweet sorghum has several advantages, such as short production cycle, and mainly, drought stress tolerance. Despite the economic and biotechnological relevance of the intrinsic abiotic stress tolerance of sorghum, the molecular mechanisms underlying such ability are not well elucidated, specially associating phenotypes characterization and proteomic analysis. So, the aim of this work was to determine the physiological and biochemical responses of different sorghum varieties under water deficit, and also identify and correlate differentially accumulated proteins to drought tolerance phenotype. In a greenhouse experiment, plants of varieties IPA-SF15, IPA-46742 and EMBRAPA-BR506 were cultivated for 55 days and, at this point, water deficit started by water withhold during 72 h, followed by rewatering. It was determined relative water content, photosynthesis rate, stomatal conductance, transpiration, chlorophylls, carotenoids, MDA and proline concentration, Brix^{o} degree and antioxidant enzymes activities. The proteomic analysis were performed comparing the sensitive and tolerant varieties. In general, IPA-SF15 plants had better performance under drought conditions, showing less concentrations of MDA, higher antioxidant enzymes activity, concentration of pigments, proline and photosynthesis rate in comparison with the more sensitive variety IPA-46742. In addition, the most tolerant variety accumulated more proteins of Calvin-cicle (RbcL, PGK), photosystems maintenance (HHL1), osmolyte synthesis (PAO) and secondary metabolism (AD2). On the other hand, sensitive variety translated more proteins related to ubiquitination and inhibition of photomorphogenesis (UBL1, COP1), confirming the sensitive phenotype. Taken together, results from proteomics and physiological analysis suggest that the diversity of mechanisms employed by IPA-SF15 are a clear advantage under water deficit conditions.

Keywords: Biofuels, Poaceae, C4 grass, Abiotic stress

SUMÁRIO

1 INTRODUÇÃO GERAL

O setor sucroalcooleiro se destaca no cenário econômico nacional agregando R$ 94,9 bilhões ao PIB Brasileiro, segundo levantamentos da safra 2013/14. Nesse cenário, a cana-de-açúcar (*Saccharum officinarum* L.) é a principal espécie cultivada, com área total plantada de aproximadamente 9.110,9 mil hectares, distribuídos em vários estados, mais notadamente no noroeste de São Paulo. Após a fase de colheita e processamento da biomassa, há um período de entressafra de aproximadamente quatro meses, fato que deixa as dornas das usinas ociosas, representando perda de tempo útil de produção.

Nesse contexto, o sorgo sacarino (*Sorghum bicolor* L.) surge com alternativa para fornecimento de matéria prima para produção de sucroderivados, durante a entressafra da cana-de-açúcar. Isso é possível devido o ciclo de vida mais curto, além da possibilidade de utilização da mesma infraestrutura e logística empregadas no processamento da cana. Além disso, o cultivo do sorgo é menos oneroso, pois é uma espécie mais versátil em relação a condições agronômicas, consumindo menos insumos agrícolas e, aproximadamente, 2/3 menos água comparativamente a *S. officinarum*.

O melhor uso dos recursos hídricos disponíveis é característica desejável, considerando os efeitos fisiológicos adversos relacionados a escassez de água. Diversos processos vegetais são afetados em tais condições, mais notadamente àqueles relacionados à fotossíntese e consequente crescimento e produtividade, tornando a dinâmica desses eventos importantes alvos de estudos e aplicações biotecnológicas.

A plasticidade fenotípica do sorgo está relacionada a maior tolerância a estresses de origem biótica e abiótica, principalmente à salinidade e ao déficit hídrico. Tal característica torna *S. bicolor* modelo funcional para estudos de resposta a diferentes tipos de estresses em gramíneas, caracterizando o sorgo com fonte de recursos genéticos biotecnologicamente desejáveis. O desenvolvimento de novas variedades mais tolerantes a seca é particularmente importante, devido às mudanças climáticas que diminuem áreas cultiváveis e a disponibilidade de água, provenientes de recursos fluviais e/ou pluviais. Assim, a prospecção de proteínas e genes de resposta eficiente ao déficit hídrico pode ser empregada no melhoramento assistido de plantas, acelerando o desenvolvimento de novas cultivares mais adaptadas as condições ambientais futuras.

Atualmente existem algumas variedades de sorgo sacarino desenvolvidas pela Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) e pelo Instituto Agronômico

de Pernambuco (IPA), no entanto pouco se conhece sobre os mecanismos moleculares de regulação em condições de déficit hídrico nessas cultivares. Nesse contexto, o presente trabalho objetiva determinar as repostas fisiológicas e bioquímicas de diferentes variedades de sorgo sacarino, conjuntamente as principais proteínas relacionadas a resposta à seca, utilizando análise proteômica como ferramenta. Além disso, determinar quais variedades são mais sensíveis e/ou tolerantes, bem como correlacionar as proteínas diferencialmente acumuladas com as rotas de ajuste frente ao estímulo estressor. A partir da caracterização fenotípica, as variedades mais contrastantes (mais tolerante *versus* mais sensível) foram utilizadas para determinação do proteoma diferencial.

2 REVISÃO DE LITERATURA

2.1 SORGO SACARINO E O SETOR SUCROALCOOLEIRO

Sorghum bicolor é uma gramínea pertencente à família *Poaceae*, tribo *Andropogoneae*, assim como o milho (*Zea mays*) e a cana-de-açúcar (*Saccharum* spp). Possui grande eficiência fotossintética devido ao metabolismo C4, produzindo quantidades de matéria verde mesmo em condições não ideais (REGASSA; WORTMANN, 2012; AMADUCCI et al., 2016). Nativa de regiões semiáridas africanas e do sudeste asiático, é conhecido como o "camelo dos cereais" e também como "cana do deserto" devido a tolerância intrínseca a estresses abióticos, mais notadamente à seca (VINUTHA et al., 2014). O sorgo utiliza mais eficientemente a água disponível no ambiente, necessitando de até 2/3 menos recursos hídricos para completar um ciclo produtivo quando comparado à cana-de-açúcar (ALMODARES; HADI, 2009). Em condições de menor disponibilidade de água, o sorgo é capaz de manter o status hídrico, sofrendo menos com os efeitos deletérios em relação ao milho. O investimento em maior densidade de raízes e a exploração de horizontes mais profundos do solo, podem estar relacionados a tal resposta, situação não registrada em milheiros (ZEGADA et al., 2012).

Do ponto de vista genético, o sorgo possui genoma com tamanho médio de 730 Mb e 34,496 mil genes (PETERSON et al., 2009). É um organismo diploide (2n=20) (RAKSHIT, 2016), considerado promissora fonte de recursos genéticos para o melhoramento de plantas, especialmente gramíneas de interesse econômico, devido à capacidade intrínseca de tolerar estresses de origem biótica ou abiótica, como altas temperaturas e salinização de solos (CALVIÑO; MESSING, 2012). Dessa forma, a plasticidade fenotípica e ploidia mais simples em comparação a outras gramíneas, fazem

do sorgo espécie modelo para esse tipo de abordagem (MULLET et al., 2014).Além disso, é uma espécie versátil em relação a tipos de solo e a concentração de nutrientes, preferindo substratos bem drenados e franco-arenosos, que favorecem o crescimento radicular fasciculado. Devido a menor necessidade de insumos agrícolas, o sorgo surge como alternativa viável para o cultivo em regiões com clima desfavorável as práticas agrícolas tradicionais, comuns em países em desenvolvimento (ZEGADA-LIZARAZU; MONTI, 2012).

O sorgo é comumente utilizado como forragem e na alimentação humana devido à expressiva concentração de proteínas na fitomassa e grãos (CIFUENTES et al. 2014). Também possui aplicação industrial como matéria prima na produção de cervejas sem glúten, biohidrogênio e plásticos (RAO et al., 2015), além de sintetizar moléculas bioativas, caracterizando o potencial para aplicações farmacêuticas (CARDOSO et al., 2017). Considerando a utilização e características morfológicas, as variedades de sorgo cultivadas no Brasil podem ser classificadas como granífera, forrageira e sacarina. As variedades graníferas são geralmente de porte médio-baixo (2 metros), produzem grande quantidade de sementes, que podem ser usadas na produção de silagem e etanol, a partir do amido sacarificado, e são menos propensas ao acamamento. Por outro lado, cultivares forrageiras produzem comparativamente mais biomassa, e são empregadas na alimentação animal (MAY et al., 2011; CONAB, 2017). As plantas de sorgo do tipo sacarino são mais altas que as demais, e possuem colmos suculentos com percentuais de Brixº acima de 15 %, além da grande quantidade de açúcares solúveis fermentescíveis, característica que as torna matéria prima promissora para produção de álcool combustível (DURÃES et al., 2012).

No Brasil, a espécie é cultivada desde a década de 1970, existindo atualmente área plantada de 621,9 mil hectares e produtividade média de 2667 kg/ha. Os maiores produtores nacionais são os estados Minas Gerais, Goiás e principalmente Mato Grosso (CONAB, 2016), sendo a maior parte das lavouras destinada a produção de grãos e silagem. Áreas de cultivo de sorgo sacarino ainda são inexpressivas no cenário nacional, bem como a utilização no setor sucroalcooleiro, estando restritas a pequenas produções em campos experimentais de empresas privadas (NOVACANA, 2013) e de pesquisa como a EMBRAPA.

Algumas progênies e variedades são desenvolvidas no Brasil, mais notadamente em programas de melhoramento da Embrapa Milho e Sorgo e do Instituto Agronômico de

Pernambuco – IPA. A variedade Embrapa BR506 é a mais cultivada do país, sendo principalmente empregada como sacarina. São plantas de florescimento precoce, de colmo suculento e doce, grandes quantidades de açucares fermenscíveis (sacarose 108,60 g L^{-1}), sendo resistentes ao acamamento. As variedades IPA-SF15 e IPA-46742 são forrageiras, devido a boa produção de biomassa, podendo também ser empregadas como sacarinas. São plantas com ciclo mais longo, e desenvolvidas para produção em regiões semiáridas. Na tabela 1 estão descritas mais detalhadamente as caracterísitcas das variedades supracitadas.

Devido o potencial energético e para produção de biocombustíveis, o sorgo sacarino tem se destacado desde meados dos anos de 1975, principalmente com o programa governamental brasileiro Proálcool. Com ciclo produtivo curto, de cerca de quatro meses, essa gramínea é alternativa viável para o fornecimento de substratos fermentescíveis durante o período de entressafra da cana, ou mesmo durante a renovação de canaviais brasileiros (REZENDE; RICHARDSON, 2017; BARCELOS et al., 2016). As possibilidades de colheita mecanizada, propagação por sementes, e utilização do mesmo maquinário e logística empregadas no cultivo de cana, corroboram tal possibilidade.

Como fonte de substratos para biocombustíveis, o sorgo pode ser completamente utilizado na produção de álcool ou energia, fornecendo o caldo, grãos e bagaço para produção de etanol (ZEGADA-LIZARAZU; MONTI, 2012). De forma geral, os caldos possuem entre 15 e 20 º Brix e 20 % de açúcares solúveis totais, constituídos majoritariamente por sacarose, além de glicose e maiores concentrações de frutose em relação a cana-de-açúcar (KHALIL et al. 2015). Por outro lado, a biomassa proveniente do processamento do caldo pode ser empregada na produção de etanol de 2° geração, possuindo menos lignina (REIS et al. 2016). O bagaço é constituído por celulose e arabinoxilanas, ácido urônico, galactose, glicose e traços de ramnose (QIU et al., 2017). Além disso, os grãos de sorgo contêm traços de taninos, proteínas, lipídeos e principalmente amido, substrato de maior interesse econômico, que após sacarificação enzimática, podem render em média 475 L de etanol/tonelada (BARCELOS et al. 2016).

Sob condições laboratoriais, sem considerar estudos de viabilidade econômica, estima-se que é possível a geração de 13.600 L de etanol por hectare de sorgo, utilizando-se os grãos, caldo e bagaço como substratos (BARCELOS et al. 2016). Além disso, em regiões de clima equatorial é possível o cultivo de três safras anuais, garantindo o fornecimento de combustível e energia elétrica a partir da queima do bagaço. Estima-se

que nessas condições possam ser gerados 10.000 toneladas de etanol e 30.000 toneladas de bagaço, gerando nove milhões de kW/ano, suficiente para abastecer 11.000 casas simples (ZHANG et al. 2016a).

Tabela 1. Características agronômicas de três variedades brasileiras de sorgo sacarino

Variedades		
EMBRAPA-BR506	**IPA-46742**	**IPA-SF15**
Altura média - 280 cm	Altura média - 250 a 350 cm	Altura média - 250 – 350 cm
Florescimento entre 70 a 75 dias	Florescimento - 90 dias	Florescimento - 90 a 100 dias
	Ciclo Total - 120 – 130 dias	Ciclo total - 120 – 130 dias
	Eficiência de uso da água - Elevada	Eficiência média de uso de água - 290 kg água/kg de massa seca
Rendimento de massa seca - 15 a 20 t ha^{-1}	Produção de matéria seca - 10 – 15 t/ha	Potencial de Produção de matéria seca - 15–18 t/ha
	Brix do colmo - 15 a 20 %	
Colmo suculento	Colmo suculento	Colmo suculento
Resistente ao acamamento	Resistência ao acamamento - Elevada	
	Proteína bruta - 4,5 a 9,0 %	Proteína bruta - 5,0 – 8,0%
	Capacidade de rebrota - Elevada	Capacidade de rebrota - Elevada
	Utilização - Silagem e forragem verde	Utilização - corte e silagem
Rendimento de colmos (TCH) - 40 a 60 t ha^{-1}	Produção de matéria verde - 30 – 60 t/ha	Potencial de Produção de matéria verde - 40 a 60 t/ha
Reação a doenças:		Resistência ás pragas e doenças - Elevada
Antracnose - moderadamente suscetível		
Ferrugem - moderadamente suscetível		
Helmintosporiose - moderadamente resistente		
Perfil de açucares:		
Sacarose (g L^{-1}) – 108,60		
Glicose (g L^{-1}) – 44,5		
Frutose (g L^{-1}) – 19,5		
ART (g L^{-1}) – 172,5		
Brix (ºB) – 20,9		

Adaptado de Silva et al (2008), Seagri-DIPA/ Embrapa (2012).

Apesar das potencialidades da cultura do sorgo sacarino, não há participação relevante no mercado de biocombustíveis brasileiro atual, e alguns fatores podem estar relacionados a tal situação, como a dificuldade de obtenção de sementes de qualidade, padronização de métodos adequados de estocagem do material colhido, e o desenvolvimento de variedades de alta produtividade (ZEGADA-LIZARAZU; MONTI, 2012). Adicionalmente, levantamentos sugerem que a produtividade média de etanol proveniente de variedades brasileiras ainda está abaixo dos 3000 L por hectare, valores considerados economicamente inviáveis (MAY et al., 2012). Estudos de viabilidade econômica sugerem a necessidade de incrementos na produtividade do sorgo sacarino para definitiva implementação da cultura, mesmo como complemento a cultura canavieira (REZENDE; RICHARDSON, 2017).

2.2 RESPOSTAS FISIOLÓGICAS EM PLANTAS SOB DÉFICIT HÍDRICO

As plantas são organismos sésseis e habitam os mais diversos ambientes na biosfera, onde estão expostas a distintas condições edafoclimáticas, que muitas vezes são adversas a manutenção da homeostase (SHINOZAKI et al., 2015). A escassez de água, seja por causas geoclimáticas ou por ação antrópica, tem se tornado evento comum ao longo dos últimos anos (WMO, 2007), aumentando as áreas de desertificação ao redor do planeta, e reduzindo as potenciais áreas de cultivo, fato que pode afetar a produção de alimentos para humanos e animais (GOHARI et al., 2013). Após a década de 1950, estima-se notável aumento de regiões com risco de perda de condições agronômicas ou de desertificação, como nordeste da China, mediterrâneo europeu e o sudeste argentino (SPITONI et al., 2014). No Brasil, o sertão do Nordeste possui clima semiárido e nele se convive sob condições de escassez hídrica, com índices pluviométricos anuais que variam entre 300-500 mm (MOURA et al., 2007). Nesse ambiente, plantas nativas possuem adaptações morfofisiológicas que permitem desenvolvimento e reprodução, porém espécies exóticas cultiváveis tendem a sofrer mais em tais condições adversas, causando prejuízos aos produtores (IBGE, 2017).

Os eventos de resposta a seca em plantas podem ser classificados em pelo menos três tipos: escape, evitação e tolerância. O escape está relacionado a capacidade da espécie de antecipar seu ciclo de vida frente ao estímulo estressor. Os mecanismos de evitação são uma resposta transiente, afim de se impedir os potenciais danos, e tal situação pode ser observada com maior crescimento de raízes, diminuição da biomassa na parte aérea ou

abscisão foliar. Cessado o estresse, o organismo volta as condições homeostáticas, e de forma geral, são ajustes não efetivos em longo prazo. Por outro lado, espécies tolerantes tem habilidade de suportar mudanças no regime hídrico de forma perene, se utilizando de mecanismos bioquímicos e fisiológicos para tal (FAROOQ et al., 2009; YADAV et al., 2016).

Em condições de escassez de água, o potencial hídrico do solo torna-se mais negativo, dificultando a absorção pelas raízes. Adicionalmente, ocorre diminuição da transpiração devido fechamento estomático, mantendo os gradientes de pressão radicular cada vez mais similares ou superiores aos encontrados no solo (SHINOZAKI et al., 2015; KUMAR et al., 2017). Com as mudanças no *status* hídrico, várias consequências podem ser registradas, e processos metabólicos vitais são modificados, mais notadamente, aqueles relacionados à fotossíntese (CHAVES et al., 2009; MA et al., 2015).

O fechamento estomático diminui o trânsito de gases, diminuindo a fixação de CO_2 atmosférico, principalmente em plantas com metabolismo C3. Esse quadro é menos pronunciado em plantas C4, que inibem a atividade fotorespiratória, com fornecimento de altas concentrações de CO_2 para rubisco, localizada nas células de bainha do feixe vascular (NYOGI et al. 2015). Nesse cenário, o organismo fica sob menor disponibilidade de substratos para ontogênese normal dos tecidos e órgãos, que pode ser evidenciado em menor crescimento, acúmulo de biomassa e produtividade.

Em ambientes sujeitos ao déficit hídrico, a baixa disponibilidade de água não é o único fator determinante para o declínio dos processos fotossintéticos. O excesso não dissipado de energia térmica e energia luminosa também acarretam danos aos fotossistemas e biomoléculas relacionadas (ZARGAR et al., 2017). Com pouca água, o excesso de energia luminosa é dissipada de formas alternativas, podendo aumentar a formação de espécies reativas do oxigênio (EROs) (RAJA et al., 2017). Tais compostos possuem valências livres que permitem ligação a outras moléculas como proteínas, lipídeos e ácidos nucléicos, oxidando-as, causando mudanças estruturais ou degradação (IMLAY, 2013). São exemplos de EROs envolvidas em situações de déficit hídrico em plantas o peróxido de hidrogênio (H_2O_2), hidroxila (OH^-) e o radical superóxido (O_2^-). Além disso, as altas temperaturas também podem causar danos diretos a moléculas e membranas. As clorofilas, que são compostos responsáveis pela captura da energia luminosa, também sofrem degradação nesse tipo de condição, como registrado em variedades de *Oryza sativa* sensíveis à seca. Inversamente, variedades mais tolerantes tem habilidade de

manter integridade das clorofilas (SWAPNA; SHYLARAJ, 2017). Por outro lado, as EROS também atuam no processo de sinalização ao estresse, sendo tal efeito dose dependente, pois quando em baixas concentrações atuam em eventos transducionais (CHOUDHURY et al. 2013; SEWELAN et al. 2016).

Frente a tal desafio bioquímico, plantas possuem um vasto arsenal de enzimas e outros compostos capazes de inibir a ação prejudicial dos EROs. A superóxido dismutase é uma enzima cloroplastidial, que catalisa a conversão do radical O_2^- em peróxido de hidrogênio, evento que diminui os danos aos fotossistemas, garantindo mesmo que parcialmente, a manutenção do processo fotossintético (PILON et al., 2011). Conjuntamente, a ascorbato peroxidase catalisa a conversão de H_2O_2 em água e oxigênio, extinguindo os potenciais agentes oxidantes (NAKANO; ASADA, 1981; NOCTOR et al., 2017). Adicionalmente, as isoformas cloroplastidiais da enzima glutationa redutase são as mais frequentes em plantas, e atuam na redução da glutationa dissulfeto (GSSH) a glutationa (GSH), tripeptídeo com potente ação antioxidante (GILL et al., 2013). Por outro lado, moléculas como carotenóides e polifenóis também podem erradicar espécies reativas deletérias (ABDALLAH et al., 2017). Corroborando tal hipótese, rápida resposta antioxidante determinada pelas atividades das enzimas glutationa redutase, superóxido dismutase, ascorbato peroxidase e catalase estão relacionadas ao fenótipo tolerante ao déficit hídrico em cana-de-açúcar (BOARETTO, et al., 2014).

Elétrons oriundos da fase fotoquímica estão relacionados ao sistema ferrodoxina-tioredoxina, que é responsável por regular a atividade de várias enzimas, dentre elas algumas no ciclo de Calvin (NIYOGI et al., 2015). A ausência do fluxo de elétrons coordenado causa inibição de algumas delas, como a ribulose-1,5-bisfosfato carboxilase-oxigenase (RuBisCO) (MA et al., 2015). Por outro lado, o fechamento estomático ocasiona diminuição da concentração de CO_2 no mesofilo, situação que estimula a atividade de oxigenase da Rubisco, causando menor eficiência fotossintética em espécies C3 (HAGEMANN; BAUWE, 2016). Tal situação inexiste em plantas de metabolismo C4 pois, devido a carboxilação em intermediários de quatro carbonos, e localização mais interna dos tecidos fotossintetizantes. As concentrações de CO_2 permanecem mais altas nos sítios próximos a Rubisco, mitigando a atividade fotorespiratória (MILLAR et al., 2015).

O trânsito de elétrons pelos fotossistemas que se traduz na conversão de energia luminosa em energia química é denominado *quenching* fotoquímico. Contudo, sob

escassez hídrica a energia luminosa excessiva e não utilizada pode ser capturada por outros sistemas moleculares, afim de mitigar os potenciais danos, evento conhecido como *quenching* não fotoquímico (NIYOGI et al., 2015). Em tais circunstâncias, moléculas como xantofilas e carotenóides funcionam como aceptores, registrando-se incremento de concentração significativo (PALLIOTTI et al., 2015). Por outro lado, dependo da espécie, outros compostos podem atuar nesse processo como antocianinas, polifenóis e flavonóides (DEL-CASTILHO-ALONSO et al., 2016). Estes últimos têm efeito fotoprotetor, e também aliviam os danos causados pela radiação UVB, estresse concomitante em ambientes sujeitos à seca.

Com as condições edafoclimáticas desfavoráveis, induzindo a perda de água constantemente, se faz necessário ajustes no gradiente osmótico tanto na parte aérea como nas raízes. Pelo menos duas grandezas determinam o fluxo de água em plantas: 1- o potencial de pressão, que está relacionado a interação da água com as partículas do solo e consequente absorção pelas raízes, e 2- potencial osmótico determinado pela concentração de solutos no ambiente intracelular (HOLBROOK, 2013). O ajuste osmótico pode ser realizado com a síntese de moléculas denominadas solutos compatíveis, que atuam diminuindo o potencial hídrico, sem quebrar as conchas de hidratação de compostos celulares, caracterizando o potencial osmoprotetor (LISAR et al., 2012; SHINOZAKI et al., 2015). Polióis, carboidratos solúveis, aminoácidos como prolina e citrulina são exemplos de solutos compatíveis sintetizados por plantas frente ao déficit hídrico, porém a quantidade e diversidade variam de acordo com a intensidade e duração do estresse, além da espécie em questão (BURG; FERRARIS, 2008; MOROSAN et al., 2017; ZANDALINAS et al., 2017).

As respostas frente ao déficit hídrico e outros tipos de estresse são intermediadas pela ação de vários hormônios que atuam como sinalizadores após percepção do estímulo agressor, além de serem efetores em vários mecanismos de ajuste (ZHU, 2016; RAJA et al., 2017). Considerando o estresse hídrico, o ácido abscísico (ABA) é um dos fitohormônios mais responsivos nesse tipo de condição. É sintetizado nas raízes, órgão onde as mudanças na disponibilidade hídrica são mais frequentemente percebidas. Nessas circunstâncias o ABA é translocado via xilema para parte aérea onde atua entre outros processos, no fechamento estomático, diminuindo a transpiração diretamente e a fotossíntese indiretamente (GEPSTEIN, 2013). Além disso, o ABA também pode ser

sintetizado diretamente na parte aérea (SHINOZAKI et al., 2017), e está também relacionado ao ajuste osmótico alterando a síntese de prolina (PER et al., 2017).

Por outro lado, as citocininas (hormônios relacionados a vitalidade celular) estimulam atividade mitótica e fotossintética, e tendem a ser menos sintetizados em estresses mais prolongados, diminuindo a razão entre parte aérea/parte subterrânea, situação que favorece o crescimento radicular e a busca de água em horizontes mais profundos do solo (LI et al. 2016).

2.3 EVENTOS MOLECULARES EM PLANTAS SOB DÉFICIT HÍDRICO

Alterações morfológicas, modificações na atividade fotossintética e ajuste osmótico podem ser consideradas variações fenotípicas de resposta a seca. Ao mesmo tempo, sumarizam imbricado arranjo molecular que permite diversas alternativas de regulação em tais circunstâncias, mecanismos que justificam a plasticidade fenotípica em diferentes espécies, sobretudo em plantas. As estratégias moleculares de enfretamento do déficit hídrico são variadas, e têm sido alvo de estudos ao longo dos últimos anos, visando, entre outros fins, o melhoramento genético de plantas (KHAN et al., 2015; LAVANIA et al., 2015; KHURANA et al., 2017).

Um dos principais alvos são os hormônios reguladores, mais notadamente ácido abscísico, etileno e as citocininas. Os níveis de ABA regulam processos bioquímicos na parte aérea e nas raízes, além de alterar a expressão genes por intermédio de receptores e fatores de transcrição específicos (BASU; RABARA, 2017). Nesse contexto, existem pelo menos dois tipos mecanismo sinalizadores: 1- dependentes de ABA e 2- independentes de ABA. A percepção do estímulo ocorre devido a mudanças no turgor e na conformação na parede celular ativando receptores (YOSHIDA et al., 2014). Posteriormente, a transdução de sinal ocorre por oscilações nos níveis citoplasmáticos de Ca^{2+} e ativação de quinases citoplasmáticas, como quinases dependentes de Ca^{2+}. Essas proteínas fosforilam fatores de transcrição específicos, ativando genes que contribuem para os ajustes necessários. Genes com sequencias promotoras do tipo ABRE (*ABA binding responsive elements*) têm níveis de expressão alterados em função das mudanças de concentração de ABA, e tal processo é mediado por fatores de transcrição do tipo ABF (*ABRE binding factors*). Por outro lado, genes que possuem sequencias promotoras do tipo DRE (*Dehydration response elements*) também estão relacionados a repostas ao déficit hídrico, porém independem da ação do ABA (YOSHIDA et al., 2014;

SHINOZAKI et al., 2017), como processos mediados por proteínas DREB (*Dehydration responsive elements binding*), que entre outros fatores, são ativados pelo etileno.

As citocininas são grupo de hormônios que aumentam a atividade e divisão celular, e que em situações de estresse, são antagonistas do ABA. A percepção e transdução de sinal ocorre pelo mecanismo *phosphorelay* onde o grupo fosforil é transferido de um receptor ao doador (que pode ocorrer em diferentes domínios de uma mesma proteína), ativando a cascata de sinalização (PEKAROVA et al., 2016). Os principais receptores membranares desse hormônio são do tipo AHK (*Arabidopsis histidine kinases*) que fosforilam proteínas AHP (*Arabidopisis histidine transfers*) e posteriormente ativam fatores transcricionais ARR (*Arabidopisis response regulators*) transcrevendo os genes responsivos. Aparentemente, pelo menos duas classes de proteínas são comuns a sinalização via do ABA e das citocininas: os receptores AHK 1 e os reguladores de resposta do tipo ARR-A. Essas moléculas estão relacionadas a inibição da ação das citocininas, o que corrobora os efeitos do ABA em plantas sob déficit hídrico (HA et al. 2012). Por outro lado, os níveis de citocininas podem estar aumentados em períodos de estresse curto, fato evidenciado por exemplo pela maior expressão de genes da enzima isopentenil transferase, principal proteína de sintetização *de novo* de citocininas (ONETO et al., 2016). Vale salientar que a especificidade das proteínas bem como a interação com os fitohormônios variam de acordo com espécie, órgão e itensidade do estresse.

Os efeitos mediados por hormônios ou pela ação direta do estímulo estressor, afetam vários processos metabólicos. Em relação a fotossíntese, os efeitos negativos da escassez hídrica e outros tipos de estresse podem ocasionar degradação dos fotossistemas (ARENA et al., 2017), que são constituídos por membranas, complexos proteicos e pigmentos (NIYOGU et al., 2015). Dessa forma, a manutenção dessas estruturas é imprescindível para sobrevivência e reprodução da planta. Genótipos tolerantes podem expressar mais certos genes plastidiais, como os da ascorbato peroxidase, NADH desidrogenase K, proteína do centro de reação Fe-S, dehidrinas e proteínas ribossomais, que provavelmente auxiliam no turnover proteico. Esses eventos estão relacionados ao uso mais eficiente da água e manutenção fotossintética (RUIZ-NIETO et al. 2015). Por outro lado, plantas com metabolismo C4 possuem vantagens em situações de seca, devido à diferenças anatômicas e bioquímicas em relação a outras espécies (NIYOGI et al., 2015). A maior expressão dos genes da fosfoenolpiruvato carboxilase e piruvato ortofosfato diquinase em plantas de arroz transgênicas, favoreceu os processos fotossintéticos em condições de

estresse hídrico severo, em comparação a indivíduos selvagens, com metabolismo C3 padrão (GU et al., 2013). Em *Sporobolus stapfianus*, espécie que tolera dissecação, maior acúmulo de proteínas do anabolismo de carboidratos e fotossistemas pode estar relacionado ao fenótipo tolerante (OLIVER et al., 2011).

Além de modificar a expressão de certos genes para substituição das proteínas degradadas, também é crucial que o maquinário celular seja preservado. Proteínas LEA (*late embriogenesis abundant proteins*) são moléculas hidrofílicas com grande estabilidade térmica, que atuam como chaperonas, protegendo componentes celulares em situações de estresse. São proteínas de ocorrência em plantas e animais e foram primeiramente identificadas em sementes, porém são traduzidas em outros órgãos vegetais (HONG-BO et al., 2005; HAND et al., 2011). Podem ser mais traduzidas em função das concentrações de ABA (DALAL et al., 2009), e atuam evitando a agregação proteica em condições de escassez de água, preservando função (GOYAL et al., 2005). Estirpes transgênicas de *Escherichia coli* portando sequencias de proteínas LEA de *Pinustabuliformis* tiveram maior tolerância a estresse salino e ao calor, corroborando as características protetivas (GAO; LAN, 2016). Em arroz, a superexpressão do gene *Os*Em1, codificante da proteína LEA de classe 1, promoveu maior tolerância ao estresse hídrico, aumentando a sobrevivência das plantas em fase vegetativa (YU et al., 2016). Tal fenótipo só foi pronunciado em condições de déficit hídrico, não se registrando diferenças em relação a indivíduos selvagens em condições controle. Efeitos similares também foram registrados em mudas de *Medicago sativa* transformadas com o gene *Cs*LEA e cultivadas sob condições de seca e salinidade (ZHANG et al., 2016b).

Outro grupo de proteínas relacionado a respostas em plantas sob o déficit hídrico é o das proteínas de choque térmico (HSP – *Heat Shock Proteins*). Essas moléculas podem atuar como chaperonas no adequado dobramento de proteínas recém traduzidas ou em transporte, garantindo correta estrutura e função, e alguns representantes participam dos processos regulados de degradação protéica ubiquitina-proteassoma (BOZAYKUT et al., 2014). Além disso, também estão ligadas à manutenção da integridade de moléculas de RNA (TATOSYAN; KRAMEROV, 2016). Certas HSPs participam da transdução de sinais engatilhados pelo estresse térmico, ativando intermediários citoplasmáticos, e posteriormente fatores de transcrição como as proteínas DREB (OHAMA et al., 2017).

São conhecidas pelo menos cinco famílias de HSP's (HSP's 40, 70, 90, 100 e pequenas HSP's), que são classificadas segundo a massa molecular que possuem, e são

divididas em dois grupos: HSP's de alto peso molecular e HSP's de baixo peso molecular (BOZAYKUT et al., 2014). Também possuem classes de fatores de transcrição específicos (WHAIBI, 2011). Sob condições prejudiciais, maior acúmulo de proteínas de choque térmico é registrado, como em estresse salino (MUTHUSAMY et al., 2017), térmico (CHEN et al. 2014), metais pesados (ARENA et al. 2017) e déficit hídrico (GOLEBIOWKSA-PIKANIA et al. 2017; SILVA et al., 2017). Esse tipo de resposta molecular já foi evidenciado em espécies de diversas famílias (WHAIBI, 2011), indicando que tal mecanismo é conservado e crucial para manutenção do organismo sob situações deletérias.

As aquaporinas são proteínas de membrana que auxiliam no transporte de água, sendo abundantes em tecidos radiculares, estando também presentes em tecidos da parte aérea. São categorizadas segundo a localização subcelular em quatro tipos: proteínas intrínsecas de membrana plasmática (PIPs), proteínas intrínsecas de tonoplasto (TIPs), proteínas intrínsecas similares a NOD26 (NITs) e proteínas intrínsecas pequenas e básicas (SIPs) (LI et al. 2014).Auxiliam no transporte de água pela via simplástica e principalmente pela via de transporte transmembranar, aumentando a condutividade. No entanto, as aquaporinas também estão relacionadas ao transporte de nutrientes e CO_2, afetando direta ou indiretamente os processos anabólicos foliares (LI et al., 2014; FOX et al., 2017). Considerando-se os efeitos da falta de água sobre a fisiologia de plantas, alguns estudos sugerem que expressão de genes de aquaporinas são modulados em tais condições (ZARGAR et al., 2017). É provável que esses transportadores auxiliem indiretamente a fixação de CO_2 em plantas pela melhora na condutividade de água e regulação estomática, porém, efeitos diretos não podem ser descartados, sobretudo pela possível presença em membranas cloroplastidiais (BEBO et al., 2013; FOX et al., 2017).

Em um contexto geral, as diferentes formas de regulação em nível molecular abrem horizonte de possibilidades para aplicações biotecnológicas. Os milhares de anos de evolução, e mais recentemente os procedimentos de melhoramento clássico, fazem de certas espécies valiosas fontes de recursos genéticos para produção de novas variedades. É necessário o cultivo de plantas mais tolerantes às condições adversas do planeta, que possuem prognósticos não animadores para os anos futuros, sobretudo em relação a disponibilidade de água. No entanto, para possibilitar a aplicação biotecnológica, a utilização de abordagens de análise de expressão gênica funcional é recomendada, pois

permitem a identificação dos principais genes e proteínas envolvidos em um processo biológico de interesse.

2.4 EVENTOS MOLECULARES EM *S. bicolor* SOB CONDIÇÕES DE DÉFICIT HÍDRICO OU OSMÓTICO

A característica de tolerância a estresses abióticos de *S. bicolor* tem atraído atenção e esforços de pesquisadores, estudando diferentes aspectos de tal fenótipo. Apesar dos diversos trabalhos sobre respostas fisiológicas e bioquímicas em sorgo (REDDY et al. 2015; FRACASSO et al. 2016), comparativamente, poucos estudos se direcionam a elucidação dos mecanismos moleculares, sobretudo diretamente focado em proteínas.

O sorgo é espécie modelo para estudos moleculares em plantas C4 (MULLET et al. 2014), e sua instrínseca tolerância a estresses, torna modelo funcionalmente mais adequado para esse tipo de estudo em comparação a *A. thaliana*, por exemplo (NGARA; NDIMBA, 2014). Há de se condiderar que *S. bicolor* é nativo de regiões semiáridas africanas, representando milhares de anos de evolução, adaptação e seleção sob tais condições, o que faz dessa gramínea valiosa fonte de recursos genéticos para produção de variedades mais tolerantes.

Dados transcriptômicos apontam para ajustes relacionados a processos fotossintéticos, osmoregulação, metabolismo hormonal e de proteínas, com respostas variando de acordo com a variedade, órgão e tempo de estresse. A tabela 2 lista o padrão de acúmulo de diversos transcritos em *S. bicolor* sob estresses abióticos, sobretudo déficit hídrico.

Em relação a abordagens no proteoma, os dados são ainda mais escassos, e por tal motivo pesquisadores sugerem e incentivam a realização de experimentos empregando análise proteômica (NGARA; NDIMBA, 2014). Os poucos estudos existentes são mais direcionados a respostas ao estresse salino. A tabela 3 lista os principais experimentos com análise proteômica em *S. bicolor* sob déficit hídrico ou salinidade, enfatizando as principais proteínas mais accumuladas em tais condições.

Tabela 2. Transcritos differencialmente acumulados em variedades de sorgo submetidas a distintas condições de estresse abiótico

Variedade	Tratamento	Amostra	Transcritos e processos biológicos	Referências
BTx623	Osmótico	Parte aérea	Transporte (PIP2↑); Fotossíntese (Enzima Málica-NADP↑) Biossíntese de hormônios; Osmoprotetor (LEA grupo 3); Fator de transcrição (MYB 4↑);	BUCHANAN et al, 2005
	Salinidade	Parte aérea	Fotossíntese (Enzima Málica-NADP↑); Biossíntese de hormônios; Osmoprotetor (LEA grupo 3↑); Fator de transcrição (MYB 4↑);	
	ABA	Parte aérea	Transporte (PIP2↑); Fotossíntese (Enzima Málica-NADP ↑); Biossíntese de hormônios; Osmoprotetor (LEA grupo 3 ↑); Fator de transcrição (MYB 4↑);	
BTx623	Osmótico	Parte aérea	Osmoprotetor (LEA grupo 3↑); Transporte (Provável proteína de transporte ABC↑); Sinalização (Inositol-3 fosfato sintetase↑); Resposta alta intensidade luminosa; Defesa; Regulação da biossíntese de clorofila; Resposta a temperatura	DUGAS et al, 2011
	ABA	Parte aérea	Osmoprotetor (LEA grupo 3 ↑); Transporte (Provável proteína de transporte ABC↑); Resposta á alta intensidade luminosa; Biossíntese de flavonóides; Resposta á temperatura; Sinalização (Inositol-3 fosfato sintetase ↑)	
IS19453	Déficit hídrico (Leve)	Folhas	Defesa (HSP 17,4↑); Detoxificação (Citocromo P450 79A1↑ e Glutationa S-transferase 1↑); Metabolismo de aminoácidos e dos lipídios;	PASINI et al, 2013
	Déficit hídrico (Moderado)	Folhas	Defesa (HSP 17,4↓); Fator de Transcrição (bZIP1↑); Detoxificação (Citocromo P450 79A1↑ e Glutationa S-transferase 1↑); Osmoprotetor (LEA ↑); Metabolismo de Aminoácidos e lipídios;	
	Déficit hídrico (Severo)	Folhas	Defesa (HSP 17,4↓); Detoxificação (Citocromo P450 79A1↑ e Glutationa S-transferase 1↑)Fator de transcrição; Fotossíntese (Enzima Málica-NADP↑); Osmoprotetor(LEA↑ e PC5S↑); Metabolismo de aminoácidos elipídios	
R16	Déficit hídrico	Folhas	Osmoprotetor (LEA↑);Sinalização (Proteína Fosfatase 2CA↑); Transporte(Proteína de transporte ABCB19↑); Metabolismo dos Lipídios; (Enoil-CoA hidratase↑)	JOHNSON et al, 2014

	Alta temperatura	Folhas	Defesa (HSP 101↑); Detoxificação (Citocromo P450 89A2↑ e Ascorbato Peroxidase 3↑)	
	Estresses combinados	Folhas	LEA↑; Defesa (HSP 101↑); Detoxificação (AscorbatoPeroxidase 3↑); Biossíntese de poliaminas(Espermidina Sintase 3↑)	
M-81E (Tolerante)	Salinidade	Folhas	Fotossíntese(Enzima málica-NADP↑ e transcetolase↑); Metabolismo de Carboidratos (Sucrose sintetase↑, Invertase↓ e Hexokinase 7↓); Resposta estímulo	SUI et al, 2015
Roma (Sensível)	Salinidade	Folhas	Fotossíntese ↓(PsaD, PsaE, PsaF, PsaG, PsaL e PsaN); Metabolismo de Carboidratos (Invertase↓ e Hexokinase 7 ↓);	
HongkeKi (Tolerante)	Baixa temperatura Temperatura	Plântula	Fatores de Transcrição (CBFs, ERFs, AP2 ↑); Deotoxificação (Citocromo P450 CYP 709C1↑ e Citocromo P450 CYP99A1↑); Defesa (HSP ↑);	CHOPHA et al, 2015
BTx623 (Sensível)	Baixa temperatura Temperatura	Plântula	Deotoxificação (Citocromo P450 CYP 709C1↓ e citocromo P450 CYP99A1↓ e Glutationa S-transferase ↑); Metabolismo de proteina (HSP ↑); Fatores de transcrição	
IS22330 (Tolerante)	Déficit hídrico	Folhas	Detoxicação (Dehidroascorbato redutase ↑); Biossíntese de lipídios (Acil-CoA sintetase 2 de cadeia longa ↑); Fotossíntese(RuBisCO subunidade menor↓); Metabolismos de carboidratos (Frutose bifosfato aldolase↓ eFrutoquinase 2↓);	FRACASSO et al. 2016
IS20351 (Sensível)	Déficit hídrico	Folhas		
BTx623 (Sensível)	Baixa temperatura	Folhas	Metabolismo de carboidratos (Frutoquinase 1↑ eInvertase↑); Biossíntese de hormônios(9-cis-epoxicarotenóide↑ dioxigenase↑); Sinalização (Indol-3-ácido acético-amino sintetase GH 3:8↑) e Fator 4 responsivo a Auxina↑); Fitohormônios	MARLA et al, 2017
Niu Sheng Zui (tolerante)	Baixa temperatura	Folhas	Metabolismo de carboidratos (Frutoquinase 1↑ e Invertase↑) Biossíntese de hormônios (9-cis-epoxicarotenóidedioxigenase ↑); Sinalização(Indol-3-ácido acético-amino sintetase GH3:8↑)	

↑- Mais acumulado; ↓- Menos acumulado;

Tabela 3. Proteínas diferencialmente acumuladas em *S. bicolor* sob condições de estresse hídrico e salino

Variedade/genótipo	Tratamento	Órgão	Proteína ou Processos biológico	Referência
Tolerante	Déficit hídrico	Folhas	Metionina sintase↑, PEP carboxilase↑; enzima málica dependente de NADP↓; frutose-1,6-bisfosfato aldolase↓; HSP's e outras chaperonas↑	JEDMOWSKI et al. 2014
Sensível	Déficit hídrico	Folhas	Proteína de estresse mediado por ABA↑; Nucleoredoxina↑; S-adenosilmetionina sintase l↑; PEP carboxylase↑; frutose-1,6-bisfosfato aldolase↓	
MN1618	Salinidade	Folha	Metabolismo primário - GDP-mannose 3,5-epimerase 1↑, Piruvato fosfato diquinase↑, Ribulose-1.5-bifosfato carboxylase/oxygenase large subunit↑, Malate desidrogenase NADP+↑, PspB↓, ATP sintase CF1↓; Defesa - Beta-glucosidase dhurrinase-2 cianogênica↑	NGARA et al. 2012
Csv-17	Salinidade	Folha	Sistema antioxidante – glutationa-s-transferase↑, peroxidases, proteina universal de resposta ao estresse↑; Metabolismo do nitrogênio – glutamina sintase↑	SWAMI et al. 2011

↑- Mais acumulado; ↓- Menos acumulado;

2.5 ANÁLISE PROTEÔMICA

Todos os eventos biológicos macroscópicos observados na natureza são originados da interação de diversos tipos de moléculas, que estão relacionadas ao genoma de cada organismo. Até a manifestação do fenótipo, os genes são regulados de distintas formas, em processos que abrangem desde a formação do complexo transcricional, até modificações pós-traducionais de proteínas reguladoras (SUGIURA et al., 2015). Considerando-se os métodos para determinação da expressão gênica, a análise proteômica destaca-se por permitir a identificação de proteínas diferencialmente acumuladas (DAPs) em um determinado momento ou condição. Isso é particularmente relevante pois, as inferências biológicas são realizadas com base em proteínas realmente traduzidas, desconsiderando-se moléculas de RNA_m degradadas ou reguladas em eventos pós-transcricionais (HONORÉ; ØSTERGAARD, 2006; HAIDER; PAL, 2013). Além disso, todas as etapas de regulação são realizadas por intermédio de proteínas, fato que permite a compreensão de processos que envolvem desde o genoma até produtos metabólicos e processos celulares por meio desse tipo de análise. Contudo, devido a maior complexidade química e as potenciais interações metabólicas, a identificação de proteínas não consiste em tarefa simples, estando passível de ajustes metodológicos incomuns a outras abordagens relacionadas (CHANDRAMOULI; QIAN, 2009).

Como ferramenta de análise de expressão funcional, a análise proteômica tem sido alternativa empregada em estudos de diferentes áreas, desde microbiologia, patologia humana até biologia de plantas (BARBOSA-NETO et al.,2014; LIKER et al., 2009; KROL; WEIDNER, 2017). Nesse âmbito, pesquisas direcionadas à elucidação de respostas a estresses abióticos em plantas têm recebido destaque (TAN et al., 2017). Estudos desse tipo são de especial relevância devido aos prejuízos agronômicos causados por mudanças no clima. Condições de salinização dos solos, aumento na temperatura do planeta, índices de radiação UVA/UVB mais intensos e escassez de recursos hídricos, pluviais ou não, constituem desafio para produção de alimentos (WHEELER; von BROUN, 2003; WEINDL et al., 2017). Tais circunstâncias também afetam o cultivo de plantas empregadas na produção de biocombustíveis (MORROW-III et al., 2014). Dessa forma, o desenvolvimento de novas variedades capazes de tolerar estresses abióticos é determinante para viabilidade da produção agrícola nas próximas décadas. Para tal, a identificação de marcadores moleculares de respostas eficientes ou não frente a condições

deletérias é relevante, sobretudo como ferramenta empregada em melhoramento assistido de plantas (DEVI et al., 2017).

A análise proteômica consiste em um conjunto de técnicas analíticas que permitem o isolamento, separação e identificação de proteínas (SARASWATHY; RAMALINGAN, 2011). Diversas abordagens podem ser utilizadas, e variam de acordo com a sensibilidade, cobertura de análise e objetivo traçado (TAN et al., 2017). Independentemente do método de escolha, a extração do material proteico é etapa crítica e deve ser realizada afim de se obter extrato o mais limpo, concentrado e diverso possível. Assim, o método de extração deve considerar as características bioquímicas da amostra e os potenciais contaminantes, fato que geralmente acarreta otimização de protocolos (WU et al., 2014; ZHANG et al., 2017).

Análises por eletroforese bidimensional (2D-PAGE – *two dimensional eletrophoresis in denaturating polyacrilamide gel*) são rotineiramente utilizadas devido a boa reprodutibilidade e relativo baixo custo. Basicamente, consistem na separação de proteínas por ponto isoelétrico em fitas com gel imobilizado (primeira dimensão) e posteriormente em gel desnaturante de poliacrilamida (segunda dimensão) (WESTERMEIER; NAVEN, 2002). Durante a focalização ocorre migração proteica até região de carga líquida zero, correspondente ao pH nativo. Uma vez separadas em primeira dimensão, as proteínas são submetidas a eletroforese e consequente individualização por massa molecular (RABILLOUD; LELONG, 2011). Posteriormente, os géis produzidos são corados para visualização das proteínas, etapa que pode ser realizada com diferentes tipos de corantes que variam principalmente em relação a sensibilidade e manuseio. De forma geral, baseiam-se na interação entre o corante e a ligação peptídica bem como cadeias laterais, destacando-se o nitrato de prata (sensibilidade em ng) e o azul de coomassie (sensibilidade em µg) (STEINBERG, 2009).Por essa razão, análises 2D-PAGE permitem a detecção de proteínas relacionadas a genes muito expressos em detrimento daquelas que são ontogeneticamente pouco traduzidos (STEINBERG, 2009; TAN et al., 2017), porém não excluindo a detecção destes (AHMAD et al., 2017).

O resultado é uma matriz inorgânica sólida, contendo proteínas em padrão que traduz a condição biológica em estudo. Porém, a determinação dos níveis de expressão é realizada com auxílio de softwares que se baseiam na densidade de pixels de cada proteína em gel previamente digitalizado. As proteínas diferencialmente acumuladas em

tratamentos contrastantes são determinadas com respaldo estatístico, sendo os principais candidatos para posterior identificação por técnicas de espectrometria de massas. Os candidatos são excisados dos géis e digeridos com alguma protease de escolha, mais comumente tripsina. Essa etapa é crucial para análise espectrométrica, pois a tripsina realiza clivagem em resíduos de lisina ou arginina, permitindo identificação das extremidades C e N terminal (RABILLOUD; LELONG, 2011).

Nesse contexto, existem dois grandes tipos de abordagens: *top-down* e *bottom-up*. Análises *top-down* são realizadas a partir de proteínas estruturalmente intactas, sendo mais laboriosas em com baixa cobertura. São ideais para estudos de caracterização com alvos específicos e proteoformas (TOELEY; BECKER, 2017). Estudos *bottom-up* permitem identificação de proteínas a partir dos peptídeos gerados química ou enzimaticamente. Correspondem a maioria dos estudos em proteômica devido a execução mais simples, garantindo também análise de proteínas recalcitrantes e de difícil isolamento, não sendo necessário a manutenção estrutural, além de permitir automação dos processos (MAGDELDIN et al., 2014).

A espectrometria de massas é uma técnica analítica que permite a determinação da massa e estrutura de moléculas, considerando a relação entre massa e as cargas adquiridas (*m/z*). Nesse tipo de análise, amostras são ionizadas e convertidas a forma gasosa pela ação de um fluxo de elétrons, posteriormente separadas em um analisador de massas e detectadas por um componente que converte o sinal para o usuário (WESTERMEIR; NAVEN, 2002). De forma geral, as configurações de um espectrômetro de massas fundamentam-se na presença de uma fonte de ionização, um analisador de massas e um detector, porém as diferentes especificações de cada componente determinam a sensibilidade e robustez da análise (EMIDIO et al., 2015).

A determinação de proteínas por espectrometria do tipo MALDI-TOF é amplamente empregada em distintas abordagens de proteômica. Nela, amostras proteicas são ionizadas com auxílio de uma matriz orgânica, mais frequentemente o ácido alfa-ciano-4-hidroxi-cinâmico, que tem absorbância máxima em comprimento de onda do laser instalado. Após o pulso de elétrons, a amostra é instantaneamente sublimada, dessorvida da matriz e ionizada (+1), sendo então acelerada por campo elétrico e analisada em uma câmara de alto vácuo – TOF (*time of flyght*). Devido ao efeito inercial do vácuo, a energia de ionização (Vo = Vf) é equivalente para todos fragmentos, fazendo com que tempo de voo seja diretamente proporcional ao tamanho do peptídeo em análise

(BURNUM ET AL., 2008; EIDIHAMER et al., 2013). Dessa forma, a amostra é visualizada em um espectrograma contendo picos de relação massa carga que são utilizados para identificação proteica em bancos de dados de espectros como Mascot. Esse tipo de abordagem é conhecido como *peptide mass figerprint* (PMF) e permite a identificação das proteínas a partir do padrão de peptídeos formados pós-digestão tríptica (THIEDE et al., 2005).

Por outro lado, análise MALDI-TOF/TOF possibilita a fragmentação de algum peptídeo de interesse, obtendo-se a sequência de aminoácidos. Após PMF, os peptídeos são reacelerados e se "quebram" mais frequentemente na interação aminoácido, garantindo a elucidação da sequência real de resíduos. O peptídeo selecionado é denominado íon parental ou precursor, e é fragmentando em uma câmara de colisão e posteriormente resolvido em um segundo analisador de massas tipo TOF. Do ponto de vista prático, resultados provenientes da fragmentação de peptídeos são mais confiáveis, pois diminuem as chances de falsos-positivo (WESTERMEIR; NAVEN, 2002; GOGICHAEVA et al., 2007).

Ao final de todas as análises, tem-se em mãos a identificação das proteínas mais acumuladas na condição de estudo. A dedução de função pode ser feita manualmente ou com auxílio de softwares de análise de ontologia gênica que possibilitam a caracterização proteica por processo biológico, localização celular, função molecular dentre outras (DESSIMOZ; SKUNCA, 2017). Posteriormente, com tais informações, a elaboração de um modelo biológico novo para as condições testadas pode ser produzido, contribuindo com novas informações sobre processos metabólicos de interesse.

A abordagem proteômica, utilizando técnicas 2D-PAGE têm sido empregada com frequência em estudos sobre estresses em plantas como sob déficit hídrico em: *Saccharum officinarum* (VITAL et al., 2017), *Triticum aestivum* (CHENG et al., 2016), *Hordeum vulgare* (GOLEBIOWKSA-PIKANIA et al., 2017), *Zea mays* (KIM et al., 2015), *Brachypodium distachyon* (CHENG et al. 2018), *Sorghum bicolor* (JEDMOWSKI et al. 2014), *Malus domestica* (ZHOU et al. 2015), *Glycine max* (Das et al. 2016), *Arachys hypogaea* (KATAM et al., 2016); Sob estresse salino em *Saccharum officinarum* (PACHECO et al., 2013), *Sorghum bicolor* (NGARA et al. 2012), e estresse térmico em *Miscanthus sinensis* (SHARMIN et al. 2013).

3 METODOLOGIA

3.1 MONTAGEM E DELINEAMENTO EXPERIMENTAL

Foi realizado um experimento em casa de vegetação, nas dependências da Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA/CPATSA, localizada em Petrolina, Pernambuco (9º 04’04” S/40º 18’46” O), período de setembro à dezembro de 2015.

Sementes das variedades de sorgo sacarino IPA-46742, IPA-SF15 e EMBRAPA-BR506 foram gentilmente doadas pela Instituto Agronômico de Pernambuco – IPA, e colocadas para germinar em vasos contendo 10 Kg de solo nativo de *Caatinga* (argisolo vermelho amarelo, coletado da camada superior de 0-20 cm). Após desbaste, as plantas (duas por vaso) foram mantidas em casa de vegetação por 55 dias, mantendo-se a capacidade de pote, além de serem irrigadas uma vezes por semana com solução nutritiva preconizada pela FAO (IZQUIERDO, 2003). Durante o experimento, as condições médias de temperatura e umidade foram de 32,19ºC e 20,7 %, respectivamente.

Após 55 dias, as plantas foram submetidas ou não ao déficit hídrico por suspensão de rega por no máximo 72 h, com posterior reirrigação. O delineamento experimental foi inteiramente casualizado, em arranjo fatorial duplo, com três variedades de sorgo: IPA-46742, IPA-SF15, EMBRAPA-BR506; e quatro tratamentos hídricos: controle irrigado, 48 h de déficit hídrico, 72 h de déficit hídrico, e reirrigação por 24 h, em 4 repetições, totalizando 48 unidades experimentais.

3.2 ANÁLISES FISIOLÓGICAS E BIOQUÍMICAS

A taxa fotossintética, condutância estomática e transpiração foram determinadas utilizando-se um dectector infravermelho de gases (IRGA-Licor, Li 6400 XT). A massa seca foi determinada por gravimetria, após secagem da parte aérea em estufa (60 ºC), o teor Brix determinado com auxílio de refretometro, e o conteúdo relativo de água determinado pelo método de BARRS;WEATHERLEY (1962).

Para quantificação de prigmentos fotossintetizantes, amostras de folhas foram trituradas com auxílio de cadinho e pestilo e maceradas em etanol 95 % por 15 min. A

partir do extrato, a concentração de clorofilas (a,b e total) e carotenoides totais foi determinada em espectrofotômetro (LICHTENTHALER, 1987).

A concentração de prolina total foi determinada colorimetricamente pela reação de alíquotas de extrato etanólico com niidrina ácida (BATES et al. 1973), e peroxidação lipídica de membranas quantificada pela reação de substâncias ao ácido tiobarbitúrico (TBARS) (CAKMAN;HOSK, 1991).

Para determinação da atividade de enzimas antioxidantes, foi produzido extrato proteico em tampão fosfato (NOGUEIROL et al. 2015) previamente as análises. A atividade da superóxido dismutase foi quantificada pela reação de inibição da fotorredução do azul de tetrazolium (GIANNOPOLITIS;RIES, 1977). A atividade da ascorbato peroxidase foi determinada pela quantificação do peróxido de hidrogênio sequestrado na presença de ascorbato (NAKANO;ASADA, 1981).

Os dados obtidos foram submetidos a análise de variância e as médias comparadas pelo teste de tukey ($p<0,05$).

3.3 ANÁLISE PROTEÔMICA

O proteoma solúvel foliar foi extraído pelo método de WU et al. (2014), após pulverização de amostras vegetais em nitrogênio líquido. O extrato produzido foi quantificado pelo método de BRADFORD (1976), e amostras de 300 µg foram adicionadas a fitas impregnadas com poliacrilamida e imobilinas com pH variando de 3-10 (GE Lifesciences) por 24 horas. Posteriormente, a focalização isoelétrica foi realizada em plataforma Multiphor 2 (GE lifesciences), acumulando-se um total de 30000 V e a separação por massa molecular em gel de poliacrilamida desnaturante 10 % (SDS-PAGE). Os géis foram corados com azul de comassie G-250 e digitalizados utilizando-se Image scanner III (GE lifiesciences).

A partir das imagens foi determinado o acumulo diferencial de proteínas no software Image Master 7.05. Para comparação foram utilizados triplicatas dos géis dos tratamentos da variedades de sorgo mais sensível e mais tolerante ao déficit hídrico, e spots com valores de ANOVA $\leq 0,05$ e ratio ≥ 1.5 foram selecionados para identificação por espectrometria de massas.

Os spots candidatos foram excisados dos géis de digeridos com tripsina por 24h (WEBSTER; OXELEY, 2002), e os peptídeos resultantes extraídos com solução de ácido trifluoroacético, acetonitrila e água (1:4:5), e transferidos para novos tubos. O material foi seco e o *pellet* homogeinizado com matriz α-cyano matrix e analizados em espectrometro Autoflex III – MALDI-TOF (Bruker Daltonics, Inc), pelo método d*e peptide mass fingerprinting* (PMF).

Os espectros obtidos foram identificados no banco de dados MASCOT (matrixscience.com), comparando-se ao proteoma teórico de *S. bicolor*. Após identificação das proteínas, a análise de ontologia gênica foi realizada na plataforma online Mercator (plabipd.de), organizando-se os candidatos por processo biológico.

4 RESULTADOS

4.1 PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF THREE SWEET SORGHUM VARIETIES IN RESPONSE TO WATER DEFICIT

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Abstract: BACKGROUND: Sweet sorghum has great content of fermentable sugars in the stems. Itmaygrown as alternative for ethanol production during sugarcane off season, due to itsshort production cycle anddrought stress.Thus, the aim of this work was to determine the physiological and biochemical reponses of contrasting sorghum varieties under water deficit. **RESULTS:** Despite the same RWC at 72h of water withhold, the tested varieties showed different strategies and responses towards drought. IPA-SF15 plants showed less concentrations of MDA, higher antioxidant enzymes activity and photosynthesis rate in comparison to the more sensitive IPA-46742 variety. BR506 had similar performance of IPA-SF15, however, accumulated more proline and SOD units, but did not show full recover of photosynthesis after rewatering such as IPA-SF15. In contrast, IPA-46742 had the worst performance, activating lately the APX antioxidant system, resulting in higher levels of membrane peroxidation and no complete recover of *Pn*. **CONCLUSIONS:** At tested conditions, IPA-SF15 is a more tolerant variety, suffering less with imposed hydric restrictions and recovering physiological and biochemical status after rewatering.IPA-46742 is a less tolerant cultivar due to a persistent damage even at rewatering.EMBRAPA-BR506 is also tolerant but utilizes different mechanism than IPA-SF15.

Keywords: Biofuels, Poaceae, C4 grass, Abiotic stress

INTRODUCTION

Sweet sorghums are varieties of sorghum (*Sorghum bicolor* L. Moench) that accumulates high quantities of soluble sugars in the culms, becoming these plants desirable for biofuels production (1). Besides that, sorghum crops need low agronomic inputs and have an intrinsic tolerance to drought (2). It can tolerate different edaphic conditions and wheatear, preferring hot and sunny environments, due to its C4 metabolism(3, 4). These features make sorghum a promising alternative crop for food, biofuel and energy production in dry regions, such as northeastern of Brazil.

In the Brazilian sugar-alcohol market, sweet sorghum may be cultivated during sugarcane off season, maintaining the mills active during the entire year(5). In addition, the short time productive cycle (~4 months) arise the possibility to cultivate this feedstock three seasons per year, becoming sorghum as main biofuels/energy crop. Actually, sweet sorghum produces impractible yields of ethanol per hectare in the Brazilian fields (6), however, this plant is the unique grass feedstock capable to provide three sources (grains, juice and bagasse) for alcohol production. In laboratory conditions, it is possible to produce 13.600 L of ethanol per hectare (5) however, few economic and logistic adjustments are needed to reach such yields in the fields(7).

Regarding the drought tolerance, sorghum is called as “sugarcane from desert” and “the camels of the crops”. It is originated from north of Africa and it is evolutively well adapted to dry conditions(4). Among grasses, sorghum is more tolerant to hydric restrictions than sugarcane and maize. It is necessary at least 2/3 less water for sorghum complete its productive cycle, in comparison with sugarcane (8). In certain drought conditions, sorghum can maintain its physiological vitality, suffering much less than maize. In field experiments, sorghum improve its root system density and explores deep soil horizons, which may partially explain the water status maintenance (9). In addition, at the early changes in soil water potential, sorghum starts to uptake more water, even with no drastic shoots water losses and it appears that this mechanism is not shared with maize(10, 11). Despite root system avoidance adjustments, the mechanisms underlying drought resilience in sorghum are not well elucidated, especially the molecular ones. Several authors suggest that it is necessary a synchrony responses of roots, culms and leaves toward water restrictions (12, 13). High accumulation of soluble sugars in the culms and others osmotic adjustments may occur (14).

In the Brazilian sorghum scenario, few promising varieties were produced by Agronomy Institute of Pernambuco (IPA) and Brazilian Company of Agropecuary Research (EMBRAPA) however, there are no basic characterizing about these plants, mainly concerning to biomass yield and abiotic stresses ability aspects. In this context, physiological and biochemical characterization will help towards improvement of sorghum yields, which is desirable for definitive use of this species in the Brazilian biofuels market (7). Therefore, the aim of this work was to determine the physiological and biochemical responses of three different sweet sorghum varieties submitted to drought stress.

MATERIAL AND METHODS

Experimental set up and design

Seeds of three sweet sorghum varieties (IPA-SF15; EMBRAPA-BR506; and IPA-46742) were gently donated by Pernambuco Agronomic Institute – IPA – Recife/Brazil. Seeds were superficially sterilized with sodium hypochlorite – 0.05 % and then sown in pots containing 10 Kg of native *Caatinga* soil during 55 days, weekly watered with a nutritional solution preconized by FAO (15). After this period, plants were submitted or not to water deficit by complete water withhold, followed or not by rewatering 24h after drought condition. Until the beginning of drought tests, all plants were maintained well-watered considering the pot capacity. The means of temperature (ºC) and relative air humidity (%) during the drought tests were 32.19 and 20.7, respectively.

The experiment was carried out in a greenhouse localized in the EMBRAPA semiarid – CPATSA buildings in Petrolina, PE/Brazil. The experimental design was completely random with factorial arrangement: three sweet sorghum varieties: 1- IPA-SF15; 2- IPA-46742 and 3 – EMBRAPA-BR506; and three water treatments: a- well watered controls; b- maximum drought stress of 72 h; c- rewatering recovery of 24h, in four repetitions, totaling 36 experimental units. For infrared gas analyzer (IRGA) analysis, we utilized four water treatments: a - well watered controls; b - 48h of water deficit; c - maximum drought stress of 72h and d - rewatering recovery of 24h in four repetitions, totaling 48 repetitions.

Collect of plant material

At the end of the experiments, for each point of analyses (control, 72h of drought and 24h after rewatering), leaves + 2 were collected, frozen in liquid nitrogen and then stored in ultra-freezer (-80°C) until the biochemical analyses.Culms were collected in order to determine Brix° of each variety in response to hydric restriction.

Physiological and biochemical analyses

Photosynthesis (Pn), stomatal conductance (g_s) and transpiration analyses (E)

The determinations were performed with a portable infrared gas analyzer (IRGA) LI-COR– Li 6400 XT. Measurements were performed in the same leaf and plants along each point of test (control, 48h of drought, 72h of drought and 24h after rewatering).

Shoot biomass dry matter, Brix° and Relative water content

Culm Brix° was determined using refractometer. Shoots were collected and air dried until constant weight, and final herbage was determined gravimetrically. The relative water content (RWC) was determined by Barrs and Weatherley (16) method.

Photosynthetic pigments

Pigments were extracted using ethyl alcohol 95 % as solvent, after shredding samples of leaves. Concentrations of chlorophyll *a*, *b* and total chlorophyll and carotenoids were determined spectrophometrycally utilizingLichtenthaler (17) method.

Proline and membrane lipid peroxidation

Proline concentration was determined by reaction of aliquots of ethanolic extract previously obtained with acid nyhidrin (18). Cellular lipid membrane peroxidation was determined based in the thiobarbituric acid-reactive substances (TBARS) assay and thereafter quantified by spectrophotometric analysis at 532 nm (19).

Antioxidant enzymes activity

Enzymes were extracted from plant material using phosphate buffer. Samples of 200 mg were homogenized (3:1) with a solution containing 100 mM of phosphate buffer (pH 7.5), 1 mM of ethylenediaminetetraacetic acid (EDTA) and 3 mM of Dithiothreitol (DTT)(20). Total proteins were quantified by Bradford (21) assay using bovine serum albumin (BSA) as standard.Superoxide dismutase (SOD, *EC* 1.15.1.1) activity was determined by nitroblue tetrazolium (NBT) photoreduction inhibition test (22). The ascorbate peroxidase

(*APX*, *EC* 1.11.1.1)activity wasquantified by the estimation of hydrogen peroxide scavenging in the presence of ascorbate (23).

Statistical analyses

Data were submitted to analyses of variance (ANOVA) and means compared by Tukey test ($p<0.05$). Data from Proline, MDA and ascorbate peroxidase activity analyses were transformed by x square before F tests.

RESULTS

Both, water treatments and sorghum varieties had effect on all tested variables (Table 1). It was registered interactions for photosynthesis rate, stomatal conductance, foliar transpiration, lipid membrane peroxidation, antioxidant enzymes activity and concentration of proline ($p<0.05$). On the other hand, water treatment and sorghum varieties influenced independently the concentrations of chlorophyll *a*, *b*, total chlorophyll and culm Brix°. Only water treatments altered the RWC. Apart of drought stress, shoot dry mass weight was different among tested varieties. At the end of 72 hours of drought, all the plants had photosynthesis rate closer to zero. For this reason, we utilized IRGA data from control, 48 hours of drought and recovery plants for statistical analysis.

Photosynthesis (Pn), stomatal conductance (g_s) and transpiration (E)

At maximum drought stress, it was registered the lower levels of photosynthesis rate closer to zero, for all the plants. However, after water recovery, IPA-SF15 showed photosynthesis levels similar to the control plants, fact not registered for IPA-46742 and EMBRAPA-BR506 varieties (Table 2), which recovered just 57 % and 62 % respectively. Even in 48 hours after water withholding, IPA-SF15 had the higher *Pn* rate compared to other two varieties. Similar behavior was observed for stomatal conductance and transpiration (Table 2).

Photosynthetic pigments

In general, IPA-SF15 had higher concentration of chlorophyll *a*, *b* and total, and carotenoids in comparison with EMBRAPA-BR506 and IPA-46742 that did not differ

from each other (Table 4). The concentration of pigments was inversely proportional to the increasing of the water stress. On the other hand, even with reestablishment of water supply, the chlorophylls concentrations continued to decline for all plants(Table 3).

Relative water content, shoots dry matter and Brix^{o}

Among varieties, EMBRAPA-BR506 had more shoot dry matter than IPA-SF15(Table 4). Similar results were registered for Brix°: EMBRAPA-BR506 had 10.9° Brix in comparison to 7.84° Brix in IPA-SF15 and 7.82° Brix in IPA-46742 (Table 4). Major levels of ° Brix were observed for all varieties submitted to 72 hours of drought stress compared to well-watered control and recovery, that did not differ from each other (Table 3). Independently the variety, drought stress diminished the RWC from 89.20 % in well-watered control to 74.07 % after 72 h of water withholding. After 24 h of water supply reestablishment, RWC was 91.35 %, very similar to the control (Table 3).

Antioxidant enzymes activity

In control treatment, major SOD activity was registered for IPA-SF15 plants, followed by IPA-46742 and EMBRAPA-BR506 (Table 2). The levels of SOD activity decreased after 72 h of drought stress for IPA-46742 and IPA-SF15. The opposite was observed for EMBRAPA-BR506 which had higher activity at maximum drought stress in comparison to the respective control,but still lower than other varieties. No recovery was registered with rewatering, however at this situation, it was observed higher SOD activity in EMBRAPA-BR506 leaves than other varieties.

Conversely of SOD activity, there were no differences among varieties for ascorbate peroxidase activity in well-watered controls. At 72 h of drought, higher APX activity was registered for IPA-SF15 and EMBRAPA-BR506 in comparison with IPA-46742. After 24 h of rewatering APX activity was increased in IPA-46742 plants but decreased in IPA-SF15 and EMBRAPA-BR506 that did not differ from each other (Table 2).

Proline concentration and lipid membrane damage

During entire experiment, the levels of MDA remained constant in IPA-SF15 plants, independent of water treatment. In EMBRAPA-BR506 plants, 72 h of drought stress increased MDA concentration followed by a decrease in rewatering treatment. On the contrary, in IPA-46742 plants the levels of MDA increased from control to rewatering treatment, with maximum registered at rewatering (Table 2). At 72 hours of drought, IPA-

SF15 and EMBRAPA-BR506 had major concentration of MDA than IPA-46742, however, after 24h of rewatering, levels of MDA in EMBRAPA-BR506 and IPA-SF15 plants was lower than in IPA-46742. In this last treatment, MDA levels represented a double in comparison to the respective control.

More proline was registered at 72 h of drought in comparison to control and rewatering treatments, for all cultivars (Table 2). However, at 72 hours of water withholding, EMBRAPA-BR506 accumulated more proline than IPA-SF15 and IPA-46742 which did not differ from each other. At 24h of rewatering, IPA-46742 and IPA-SF15 had levels of proline similar to the control, but EMBRAPA-BR506 showed higher concentration than other plants.

DISCUSSION

The RWC was similar in all plants but decreased with drought (Table 2). As expected, along 72 h of stress, plants lost water to warm atmosphere, in similar patterns, excepting for IPA-SF15 that in 48 hours of drought had higher transpiration rate and stomatal conductance. The RWC is related to the capacity to tolerate hydric stress, and the maintenance of water in leaf tissues might be a good strategy to deal with hydric restrictions. In contrast to our results, sorghum tolerant varieties could maintain water relative content in drought situations(24). On the other hand, tolerant genotypes of rice had approximately 70 % of RWC under drought, however such plants had better physiological performance than genotypes with more water in the leaves (25). Apparently, plants can face the same abiotic conditions in a distinct manner, using differently the available water. In this experiment all varieties, plants had the same RWC at 72 h of drought and at 24 h of rewatering, but the results on metabolisms were completely different, mainly in the biochemical parameters. Sorghum is known for its capacity to uses better the available water comparatively to other species(10), and in this work, the same behavior is occurring for distinct varieties.

At maximum drought, it was registered higher Brix°, possibly resulting for the concentration of soluble sugars in the culms due to water losses. In addition, EMBRAPA-BR506 had significant more Brix than other plants, confirming its potential as saccharine variety. EMBRAPA-BR506 is an early flowering cultivar (26) and might accumulate more sugars in the culms at pre-flowering stage (9), as observed in this work. On the other

hand, IPA-46742 and IPA-SF15 produce high quantities of herbage and for this reason are considered both as fodder and saccharine varieties.

Drought stress inhibited photosynthesis in all tested plants. IPA-SF15 had better performance in all stressed times in comparison with other varieties, recovering approximately 100 % of initial photosynthesis after rehydration (Table 2). The deleterious effects of drought in photosynthetic processes are well documented in grasses (13, 25, 27), and is related to low gas influx and conductance. In this situation, the lower concentration of CO_2, impairs the reduction steps in Calvin cycle, diminishing the consumption of ATP and NADPH, and hence, the available oxidized ADP and $NADP^+$, which would be reutilized in photochemical reactions(28). In this scenario, the electrons are not employed in photochemical quenching, and might be accepted by other molecules, degrading strucutures and producing ROS. The imbalanced transit of electrons also inhibit the regulatory thioredoxin system, which is related with reduction of S-S bonds and activation of several Calvin cycle enzymes (29). Additionally, the pH of stroma becomes more acid and the environment more viscous, which might inhibit the activity of Rubisco or Rubisco activase, for instance (12, 30, 31). On the other hand, the excess of not utilized energy might be dissipated in other chloroplast molecules, like chlorophylls, photosystems proteins and phospholipids, which is also related to reduced photosynthesis (32, 33).

Based in the photosynthetic performance during drought and recovery, it seems that IPA-SF15 is more tolerant than others at tested conditions. The ability to recover photosynthesis after a period of stress may be a good indicative of tolerance, especially when compared with more sensitive species like maize (34, 35). However, it is important to be correlated with other biochemical and physiological analysis, due to different strategies to deal with drought as registered for sorghum varieties (24).

Here, it was registered lower concentration of photosynthetic pigments due drought, with no recovery after rehydration. However, IPA-SF15 had more carotenoids and chlorophylls than EMBRAPA-BR560 and IPA-46742, that partially justifies the relative superior photosynthesis. Similar results were obtained by Nxele, Klein (36)in sorghum plants submitted to 16 days of drought.

Surprisingly, IPA-SF15 recovered photosynthesis but did not the concentration of chlorophylls, indicating that these plants use more efficiently the available pigments than other varieties. Ort, Merchant (37) suggest that more efficient plants might have less pigments and photosystems to better use the available light, diminishing the side effects

of excessive captured energy. Indeed, mutant rice plants with lower concentration of pigments can use better the available CO_2 in the mesophyll, showing major photosynthetic rate and nutrient utilization when compared with wild rice plants(38). However, such results were obtained at non-drought conditions, and comparisons to stress situations must be done with parsimony. On the other hand, IPA-SF15 had more chlorophylls than other varieties, which might be related to its better photosynthethic performance.

The analysis of antioxidant enzymes showed clearly the different strategies of tested plants. SOD activity is related to response to formation of superoxide radicals in the chloroplasts. The major concentration of such radicals species occurs due to problems in photochemistry and electrons transport, manly excessive absorption of photons, conditions commonly found in drought stress (41). In this survey, just EMBRAPA-BR506 invested in SOD antioxidant system during maximum drought, suggesting increased production of $^{\cdot}O_2$ in this variety. Indeed, SOD is also localizedat thylakoid membranes, and the disruption of phospholipids due to drought might decreased the SOD activity in IPA-SF15 and IPA-46742 plants (39). In addition, Gołębiowska-Pikania et al.(42)reported low activity of SOD system in drought susceptible *Hordeum vulgare* lines. Another possibility is the action of non-enzymatic antioxidant systems, such as the accumulation of phenolic compounds in the rest cultivars(43), however, it is necessary the quantification of secondary metabolites to prove such hypothesis.

The APX activity was more pronounced at maximum drought for IPA-SF15 and EMBRAPA-BR506 and lately at rewatering in the IPA-46742 plants. APX is an enzyme that coverts toxic H_2O_2 into water and O_2, and it is very important inside chloroplast environment, detoxifying reactive oxygen species (ROS) at its origin place(39). Hence, it is possible that scavenging of excessive H_2O_2 preserves the chloroplastic structures such as membranes and photosystem proteins. On the other hand, it seems that IPA-46742 perceives and respond to oxidative stress lately in comparison with other varieties, representing a potential danger for cellular processes. In fact, drought tolerant genotypes generally use efficiently the APX antioxidant system as registered in tolerant sorghum cultivars(40).In addition, it was found a synchronism between SOD and APX activities in EMBRAPA-BR506. Such enzymes are strictally related due to H_2O_2 produced by SOD, which is substrate for APX.

For all plants, it was registered increasing on the proline concentration due to drought, nevertheless. EMBRAPA-BR506 accumulated at least 40 % more proline.

Corroborating our results, high concentrations of proline were found in African sorghum plants submitted to 16 days of drought and salinity (36). Proline is an amino acid known as osmoprotector, due to its capacity to maintain the osmotic potential without destroy the hydration shells of cellular molecules, and it is commonly accumulated in abiotic stresses such as drought(28, 44). It has been reported that higher concentrations of proline and other compatible solutes contribute to recovery of the turgor after a period of hydric restrictions (45, 46), and it is possibly more important to EMBRAPA-BR506 to deal with drought than other varieties. Such aspect is particularly interesting due to alternative forms that proline can protect plants such as chaperone and antioxidant activity (47).

The effects of drought are complex and cause many damages in the cell components. In the plants, the chloroplasts are the main organelles, being closely related to abiotic stresses due to photosynthetic reactions and consequent generation of dangerous molecules. Degradation of thylakoid membranes and the less quantity of available water, impair the anabolic processes which diminishes plant growth and productivity (12). In this context, MDA concentration is a reliable analysis to determine lipid peroxidation and the membrane damages(48), and in this work showed clear differences among varieties, and are probably related to other physiological and biochemical results found here. Major concentration of MDA was registered in IPA-46742 plants at 24 h after rewatering, indicating that these plants suffered more than others. It is possible that the damage occurred along the period of stress was higher than the plant capacity to mitigate it. It is particularly probable when it compares to APX activity, which was intense at 24 h after rewatering, suggesting a late response.

On the other hand, to EMBRAPA-BR506 had major membrane damage at 72 h but recoveredit rewatering. For this variety, it was registered increased activity of SOD and APX, and the proline levels at maximum drought, possibly to deal with oxidative stress efficiently and synchronously. On the contrary, IPA-SF15 maintained the membrane stability and possibly suffered less with imposed stress which is corroborated with data from photosynthesis analysis. The antioxidative responses might underlie less membrane degradation as reported by Chakraborty and Pradhan (49) in *Triticum spp.* cultivars, which the most tolerant plants had lower MDA concentration. Reinforcing such idea, the drought priming of *Olea europea* L. seedlings promoted increases on the antioxidant systems with no changes in MDA levels, result not registered for non-primed plants (50).

Based in the physiological and biochemical analysis, it supposes to IPA-SF15 has the ability to maintain photosynthetic apparatus, due to efficient antioxidant systems, mainly APX activity, and such features confer adequate recovery after rewatering. On the other hand, EMBRAPA-BR506 also mitigate the deleterious effects of drought but utilizing different mechanisms. Finally, IPA-46742 suffered more than other varieties, probably due to the lateness perception and response to drought outcomes. Confirming our hypothesis, Fracasso et al. (24) suggest that tolerant varieties perceive the drought damages early than sensitive ones.

CONCLUSIONS

The tested drought conditions influenced the sorghum varieties, promoting different patterns of physiological and biochemical responses. It seems that at a short period of stress, the chloroplastic metabolic process are more impacted and might determine the tolerant responses to drought. Probably, the membrane stability mediated by antioxidant systems and some osmotic adjustments maintain photosynthetic apparatus, guarantying adequate recover after rewatering. In addition, APX system is fundamental as early efficient antioxidative mechanism and might determine membrane damages. On the contrary, late perception and response to the drought is very hazardous for plants and might determine the performance in the environment. Thus, at tested condition, IPA-SF15 is the most tolerant variety, suffering less with imposed hydric restrictions and recovering physiological and biochemical status after rewatering. In contrast, IPA-46742 is the least tolerant cultivar due to a persistent damage even at rewatering. EMBRAPA-BR506 is also tolerant but utilizing different mechanism than IPA-SF15.

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Table 4. Chemical characterization of the substrate employed for cultivation of sweet sorghum varieties submitted or not to water deficit

Substrate	E.C dS.m	pH H_2O-1:2.5	O.M $g.Kg^{-1}$	**P	*K	*Na	*Ca	*Mg	*Al	H+Al	SB	B	**Cu	**Fe	**Mn	**Zn
Soil	0.23	5.7	3.3	17.0	0.33	0.07	1.0	0.7	0.0	1.6	2.1	0.19	0.5	67.9	32.7	6.1

E.C – electrical conductivity; O.M- organic matter; * cmol dm^3; ** mg dm^3

Table 2. Significance levels for studied variables considering isolated effects of water treatments (W) and sorghum varieties (S) and their interactions

Variable	**S**	**W**	**S x W**
Photosynthesis	**	**	**
Stomatal conductance	**	**	**
Foliar transpiration	**	**	**
Chlorophyll *a* concentration	**	**	NS
Chlorophyll *b* concentration	**	**	NS
Total chlorophyll concetration	**	**	NS
Foliar carotenoids concentration	**	**	NS
Foliar proline concentration	**	**	**
Lipid membrane damage (MDA)	*	**	**
Superoxide dismutase activity	**	**	**
Ascorbate peroxidase activity	*	**	**
Relative water content (RWC)	NS	**	NS
Shoot dry mass	**	NS	NS
Total Brix° in culms	**	**	NS

Significance by Tukey test: *p<0.05; **p<0.01; NS – not significant

Table 3. Photosynthetic and biochemical parameters of three sorghum varieties submitted or not to water deficit

Sorghum varieties	Photosynthesis rate (µmol CO_2 m^2 s^{-1})		
	Control	48h	Rehydration (24h)
IPA-46742	36.20 aA	1.56 bC	20.82 aB
IPA-SF15	26.88 bA	7.84 aB	23.51 aA
EMBRAPA-BR506	40.77 aA	2.34 bC	25.29 aB
	Stomatal conductance (mol of H_2O m^2 s^{-1})		
IPA-46742	0.1825 aA	0.0062 bC	0.0875 aB
IPA-SF15	0.1300 bA	0.0350 aB	0.1000 aA
EMBRAPA-BR506	0.2154 aA	0.0108 bC	0.1150 aB
	Transpiration rate (mmol of H_2O m^2 s^{-1})		
IPA-46742	6.80 aA	0.30 bC	4.01 aB
IPA-SF15	5.19 bA	1.46 aB	4.51 aA
EMBRAPA-BR506	7.78 aA	0.56 bC	5.07 aB
Biochemical Parameters			
Sorghum varieties	SOD activity (units of SOD g^{-1} DW)		
	Control	72 h	Rehydration (24h)
IPA-46742	5331.65 aA	2828.90 aB	123.38 bC
IPA-SF15	3807.54 bA	2823.97 aB	208.25 bC
EMBRAPA-BR506	1507.24 cB	1952.60 bA	608.95 aC
	Ascorbate peroxidase activity (µmol H_2O_2 min^{-1} g^{-1} DW)		
IPA-46742	0.83aC	1.09 bB	1.42aA
IPA-SF15	0.92aB	1.58 aA	0.92bB
EMBRAPA-BR506	1.07aB	1.54 aA	1.16bB
	TBARS (µmol MDA g^{-1} DW)		
IPA-46742	2.19 bB	2.46 bB	5.17 aA
IPA-SF15	3.25 aA	3.51 aA	3.20 bA
EMBRAPA-BR506	2.20 bB	3.20 abA	2.84 bAB
	Proline (µg g^{-1} DW)		
IPA-46742	4.72 aB	11.24 bA	5.20 bB
IPA-SF15	6.08 aB	11.66 bA	5.06 bB
EMBRAPA-BR506	5.30 aC	16.36 aA	7.35 aB

Means followed by the same letter, lowercase in the column and uppercase in the row, do not differ by Tukey test (p<0.05).

Table 3. Foliar pigments, culm brix and relative water content among different water treatments, independently of sorghum varieties

Water treatments	Chlorophyll *a* (mg g^{-1} DW)	Chlorophyll *b* (mg g^{-1} DW)	Total chlorophyll (mg g^{-1} DW)	Carotenoids (mg g^{-1} DW)	Brix°	RWC(%)
Control	3.68 a	2.39 a	6.08 a	329.09 a	8.53 b	89.20 a
72 hours of drought stress	2.60 b	1.79 b	4.39 b	273.08 b	10.43 a	74.07 b
Recovery 24h after drought stress	1.52 c	1.03 c	2.55 c	159.03 c	7.58 b	91.35 a

Means followed by the same letter do not differ by Tukey test (p<0,05).

Table 4. Foliar pigments, culm brix and shoot dry matter in different sorghum varieties, independently of water treatments

Sorghum varieties	Chlorophyll *a* (mg g^{-1} DW)	Chlorophyll *b* (mg g^{-1} DW)	Total chlorophyll (mg g^{-1} DW)	Carotenoids (mg g^{-1} DW)	Dry matter (g)	Brix°
IPA-46742	2.50 b	1.65 b	4.15 b	253.11 ab	14.80 ab	7.82 b
IPA-SF15	2.90 a	1.94 a	4.84 a	270.93 a	13.63 b	7.84 b
EMBRAPA-BR506	2.41 b	1.61 b	4.03 b	237.15 b	16.51 a	10.90 a

Means followed by the same letter do not differ by Tukey test (p<0,05).

Table 5. Physiological and biochemical performance and categorization of three sweet sorghum varieties based in previous ANOVA analysis and Tukey test

Sorghum Variety	*Pn*	g_s	*E*	RWC	DM	Chl *a*	Chl *b*	Total Chl	Car	Brix°	Prol	MDA	APX	SOD	Total
IPA-SF15	*	*	*	*	-	*	*	*	*	-	-	*	*	-	10
EMBRAPA-BR506	-	-	-	*	*	-	-	-	-	*	*	-	*	*	6
IPA-46742	-	-	-	*	-	-	-	-	-	-	-	-	-	-	1

Pn= photosynthesis; g_s= stomatal conductance; *E*= transpiration; RWC= relative water content; DM= dry matter; Chl *a*= chlorophyll *a*; Chl *b*= chlorophyll *b*; total chl= total chlorophyll; Car= carotenoids; Brix°= Brix degree; Prol= proline; MDA= lipid membrane peroxidation; APX= ascorbate peroxidase; SOD= superoxide dismutase.
- comparative worst performance among varieties; * comparative best performance among varieties.

4.2 2D BASED PROTEOMICS REVEAL DIFFERENT MECHANISMS TOWARDS DROUGHT STRESS ADJUSTMENTS IN CONTRASTING SWEET SORGHUM (*SORGHUM BICOLOR*) VARIETIES

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Abstract: *Sorghum bicolor* is C4 plant important in human and livestock uses, besides to be feedstock for energy and ethanol production. In addition, such species has an intrinsic tolerance to abiotic stresses, being more adaptable to drought conditions than other grasses. Thus, *S. bicolor* is a desirable genetic resource for grasses breeding programs, and proteomic tools are suitable to find functional drought tolerance biomarkers. Based in physiological analysis, we determined the differentially accumulated proteins (DAPs) in two contrasting sorghum varieties submitted to 72 h of water deficit, in a greenhouse experiment. All plants suffered with drought, but only the tolerant variety SF15 had higher APX activity, lower MDA concentrations and complete recovery of photosynthesis after rewatering. In addition, SF15 accumulated more proteins of photosynthesis, polyamine metabolism and ABA signaling. Oppositely, the sensitive variety translated more proteins of ubiquitination, protein re-folding and skotomorphogenesis processes, pointing out its sensibility. Seeing the protein accumulation profile of the two sorghum varieties, it seems that the higher diversity of mechanisms employed to cope with drought in tolerant variety represents a clear advantage. We suggest the HHL1 and COP1 as candidates for efficient and inefficient water deficit tolerance biomarkers, respectively.

Key words: gel-based proteomics, semiarid, C4 grass, abiotic stress.

1. Introduction

The world climate changes are altering the dynamics of several biosphere process, becoming a serious problem for mankind, especially due to crop yield and livestock losses. Such changes impact the soil quality, decreasing its nutritional, physical and biological proprieties. Altogether, the more frequently occurrence of different abiotic stress contribute to deteriorate the farming conditions, fact that might reduce the availability of food and increase the prices (Wheeler and von Broun, 2011; Weindl et al. 2017). Water restriction is the main factor to impair plant yield, and it has been more frequent, especially in semi-arid and other dry regions, which are predominantly localized in developing places.

In this context, the development of new plant varieties, more adapted to non-ideal conditions are preconized, and it has been a challenge in food production scenario (Wheeler and von Broun, 2011). Plant species with useful genetic background are fundamental for such biotechnological development, mainly those tolerant to abiotic stresses. *Sorghum bicolor* (Moench L.) is an important grass for food, fodder, ethanol and energy production (Almodares and Hadi, 2009; Vinutha et al. 2014). In addition, it has intrinsic tolerance to biotic and abiotic stress (Calviño and Messing, 2012), being more tolerant than other grasses (Almodares and Hadi, 2009; Zegada-Lizarazu et al. 2012). In ethanol market scenario, farmers may cultivate *S. bicolor* during sugarcane off season due to short life cycle (120 days) and lower agricultural input necessities, using at least 1/3 of water (Almodares and Hadi, 2009). Such species is known as “the camel of the crops” by Indian folks due to its drought tolerance, and for such reason, it is more cultivated in drier regions (Vinutha et al. 2014).

S. bicolor is a molecular model species for other grasses with C4 metabolism, and several authors also suggest this monocot as model for functional studies about abiotic stresses (Mullet et al. 2014; Ngara and Ndimba, 2014a), being more adequate than *Arabidopsis thaliana*, which has no proved tolerance towards salinity or drought for instance. Sorghum is native from dry hot areas such as northwest of Africa, which means thousands of years of adaptation and selection in a harmful environment. Thus, to understand molecular aspects and gene expression patterns in *S. bicolor* under abiotic conditions might be useful for discovery of new biomarkers potentially employed in plant breeding, mainly economical important grasses.

Since the sequencing of *S. bicolor* genome, which has approximately 730 Mb of total genome and 34,496 mil genes (Paterson et al. 2009), several surveys of functional gene expression have been done, mostly approaching the transcriptome (Buchanan et al. 2005; Dugas et al. 2011; Pasini et al. 2013). On the other hand, proteomic researches with *S. bicolor* under osmotic stresses are still scarce (Jedmowisky et al. 2014; Ngara et al. 2012), and for this reason, sorghum scientific community has been suggesting the relevance of such molecular approach (Ngara and Ndimba, 2014a, b).

Proteomics comprises a group of molecular tools, commonly used to identify patterns of regulation in different biological situations (Saraswathy and Ramalingan, 2011). In contrast to other molecular approaches, such as diversity genetic microsatellites and transcriptome analyses, the identification of proteins may be considered a better functional way to determine gene expression (Honoré and Østergaard, 2006; Haider and Pal, 2013). In this context, it is possible to understand pathway interactions and other events do not deductible based in genome sequences or transcripts. Moreover, proteins are closer to the phenotype events which corroborate proteomics as a powerful biological tool. Often, researchers use proteomic approaches to understand responses to salinity (Pacheco et al. 2013), cold (Hu et al. 2017), warm (Timbaud et al. 2016) and other stresses in economically important crops.

The comparisons among contrasting plant varieties under abiotic stress have been widely used by different authors (Pacheco et al. 2013; Marla et al. 2017) and it is an interesting method to understand the strategies underline the tolerate to harmful conditions (Zhou et al. 2015; Urban et al. 2017), especially when the varieties are well characterized in a physiological point of view (Ngara and Ndimba, 2014a). Thus, the aim of this work was to investigate leaf differential accumulated proteins (DAPs) in physiologically contrasting sweet sorghum varieties under water deficit. We tested the hypothesis that the three varieties are able to use distinct strategies to cope with drought conditions, which might point out metabolic pathways related to efficient and/or inefficient adjustment strategies.

2. Material and Methods

2.1 Greenhouse experiment set up

The experiment was carried out in a greenhouse localized in the EMBRAPA Semiárido – CPATSA buildings in Petrolina, PE/Brazil (9º 04’04” S/40º 18’46” W). Seeds of three sweet sorghum varieties were superficially sterilized with sodium hypochlorite – 0.05 %

and then sown in pots containing 10 Kg of native *Caatinga* soil (red yellow argissoil collected from 0-20 cm upper layer) during 55 days. Plants were watered with a nutritional solution as recommended by FAO (Izquierdo, 2003) once week. After this period, plants were submitted or not to water defict by complete water withhold, followed or not by rewatering 24h after drought condition. Until the beginning of drought tests, all plants were maintained well-watered considering pot capacity. The means of temperature (ºC) and relative air humidity (%) during the drought tests were 32.19 and 20.7, respectively.

The experimental design was completely random with factorial arrangement with two sweet sorghum varieties – 1- IPA-SF15and 2- IPA-46742 and three water treatments: a- well watered controls; b- maximum drought stress of 72 hours; c- rewatering of 24h after maximum drought, in four repetitions, totaling 24 experimental units.

2.2 Physiological determinations:

To classify the tested varieties in response towards drought, it was determined relative water content (RWC) (Barrs and Weatherley, 1962), photosynthesis rate, activity of ascorbate peroxidase (APX) antioxidant enzyme and malondialdehyde (MDA) production. Photosynthesis rate (*Pn*) was determined utilizing a portable infra-red gas analyzer (IRGA) LICOR – Li 6400 XT, starting at 8 am during a fully sunny day. In addition, MDA concentration which represents the levels of membrane degradation was colorimetrically quantified (Cakmak and Horst, 1991) as well as the activity of ascorbate peroxidase (Nakano and Asada, 1981). All data were submitted to analysis of variance (ANOVA) and means compared by Tukey test ($p<0.05$) utilizing Assistat software (Version 7.7).

2.3 Protein extraction

Two hundred mg of plant material was pulverized in a mortar with liquid nitrogen and pestle. Thereafter, samples were homogenized with trichloroacetic acid solution 10 % (w/v) and transferred to Eppendorf tubes. After vigorous stirring, samples were centrifuged at 10,000 g for 10 min. Supernatant was discarded and pellet was mixed with 500 µL of extraction buffer (sucrose 0.7 M, Tris-HCl 0.5 mM – pH 7.5, EDTA 50 mM and potassium chloride 0.1 M and DTT 15 mM) and 500 µL Tris-HCl saturated-phenol (pH 8.8). The mixture was stirred for 30 min and centrifuged at 10,000 g for 15 min and the organic upper phase was collected. Phenol extract was precipitated overnight in a

solution of 0.1 M ammonium acetate in methyl alcohol, and then centrifuged to obtain a rich protein pellet (Wu et al. 2014). Proteins were resuspended in urea buffer (8 M) and quantified by Bradford (1976) assay.

2.4 Two-dimensional gel electrophoresis (2D-PAGE)

Immobiline gel strips pH 3-10 (GE Healthcare) were overnight rehydrated with 300 µg of proteins homogenized in a rehydration solution (Tris-HCl 50 mM – pH 8.8; urea 7M; thiourea 2M; DTT 70 mM; Triton X - 0.4 %; CHAPS - 2 % and amphollines 0.08 %). The isoelectrical focusing was performed in three phases, accumulating a total of 30,000 V until the end of the analysis. After focusing, strips were washed with equilibration buffer for reducing and alkylation of the samples. Proteins were separated in SDS-PAGE 10 % and 2D gels were stained with a colloidal Comassie blue solution 1 %.

2.5 Differential accumulation analysis

Gels were digitalized using a scanner (Image Scanner III GE Healthcare) and software (Lab Scan 6.0 GE Healthcare) and the produced images were analyzed in ImageMaster Platinum 7.0.5 software (GE Heathcare). Three comparative technical replicates were performed, all comparing 72 h of drought conditions from tolerant versus susceptible varieties. Statistically significant spots ($p \leq 0.05$) were selected for spectrometry analysis.

2.6 MALDI-TOF MS analysis and protein identification

Spots were excised from gels and proteins were digested with trypsin according to Webster and Oxeley (2002) method. The resulting peptides were obtained with an extraction solution (10 % TFA, 40 % ACN) and then homogenized with α-cyano matrix and analyzed in an Autoflex III – MALDI-TOF spectrometer (Bruker Daltonics, Inc), based on peptide mass fingerprinting. Protein identification was performed utilizing an in-house licensed Mascot online software against a putative proteome of *S. bicolor* and other Poaceae species from Uniprot database (www.uniprot.org). In Mascot search engine, oxidation of methionine and carbamidomethylation of cysteine were set as variable and fixed modifications, respectively, allowing one miscleavage and maximum 1 Da peptide error tolerance.

2.7 Bioinformatic analysis

Proteins were categorized by biological process in Mercator (www.plabipd.de/portal/mercator-sequence-annotation) search engine and the probable metabolic interactions utilizing String database (https://string-db.org), with *Arabidopsis thaliana* as model species. Additionally, due to poor characterization of sorghum proteins in Uniprot database, the subcellular location of the identified DAPs was determined utilizing the online software DeepLoc 1.0 (http://www.cbs.dtu.dk/services/DeepLoc/), and the function of proteins predicted with Prosite tool, considering the conserved domain.

Heat map of all comparisons was produced from the %Volume distribution of DAPs spots, utilizing Genesis software 1.8.1, and the hierarchical clustering was based in Euclidean distance test and UPGMA. The enrichment tests were performed in String database, considering false discovery rate (FDR) ≤ 0.05.

3. Results

3.1 Physiological analysis

At control conditions, IPA-SF15had lower photosynthesis rate than IPA-46742. During maximum stress, all varieties reduced photosynthesis to zero, but recovering it after 24 h of rehydration. IPA-SF15was the only variety to reestablish the original levels of *Pn*, what was not detected for IPA-46742, that showed approximately a half of the control *Pn* (Table 1).

As expected, all varieties increased the APX activity during water restrictions. However, IPA-SF15presented higher APX activity than IPA-46742 at 72 h of drought. At recovery, IPA-SF15decreased APX activity to similar levels to the control, while IPA-46742 kept increasing its activity, reaching the highest levels observed along entire experiment (Table 1).

The levels of MDA remained the same during the whole experiment for IPA-SF15,however, in IPA-46742, the concentration of MDA remained unaltered at 72 h of drought but drastically augmented after rewatering (Table 1).

Table 1. Physiological and biochemical parameters of three sorghum varieties submitted or not to water deficit

	Photosynthesis rate (µmol CO_2 m^2 s^{-1})		
Sorghum varieties	Drought stress		
	Control	72h	Rehydration (24h)
IPA-46742	36.20 aA	0.00 bC	20.82 aB
IPA-IPA-SF15	26.88 bA	0.00 bC	23.51 aA

	Ascorbate peroxidase activity ($\mu mol\ H_2O_2\ min^{-1}\ g^{-1}$ DW)		
IPA-46742	0.83aC	1.09 bB	1.42aA
IPA-IPA-SF15	0.92aB	1.58 aA	0.92bB
	TBARS (μmol MDA g^{-1} DW)		
IPA-46742	2.19 bB	2.46 bB	5.17 aA
IPA-IPA-SF15	3.25 aA	3.51 aA	3.20 bA

Means followed by the same letter, lowercase in the column and uppercase in the row, do not differ by Tukey test (p<0.05).

Table 2. Relative water content (RWC) among different water treatments, independently of sorghum varieties

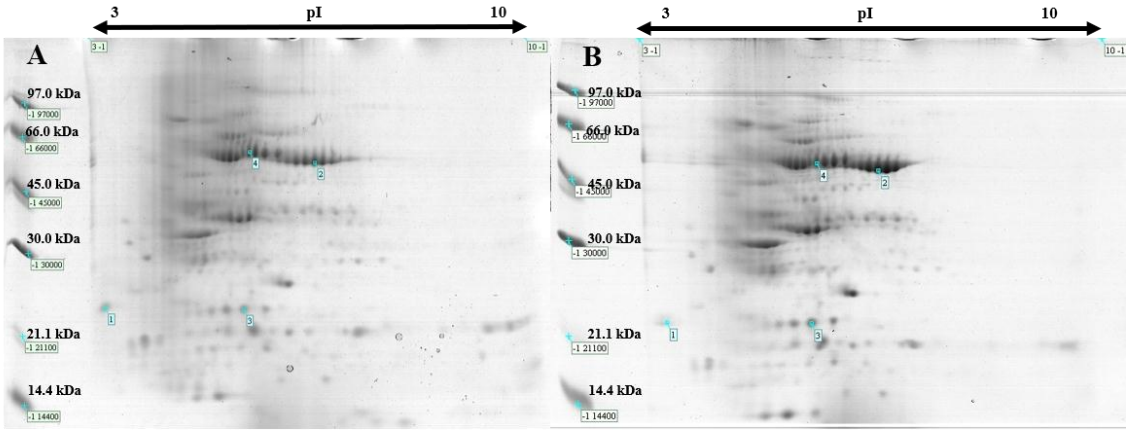
Water treatments	RWC(%)
Control	89.20 a
72 hours of drought stress	74.07 b
Recovery 24h after drought stress	91.35 a

Means followed by the same letter do not differ by Tukey test (p<0,05).

3.2 Proteomic analysis

A total of 962 spots were detected in all samples used in comparison. Out from that, 24 statistically significant spots were identified. Such proteins are involved in 14 different biological processes, mainly photosynthetic pathways and protein metabolism. IPA-46742 accumulated more proteins related to protein metabolism, response to stress and transcription factors. In addition, proteins related to development and redox responses were exclusively identified in such variety(Table 4, Figure 1).

On the other hand, the tolerant sorghum variety accumulated much more proteins of photosynthetic pathways, cell wall and exclusively proteins of polyamine, amino acids and secondary metabolisms (Table 4, Figure 2).



A- IPA-SF15

B- IPA-46742

Figure1. 2D-PAGE gels from constrating sorghum varities IPA-SF15 and IPA-46742

Corroborating GO categorization, enrichment analysis showed the statistical significance of carbon related pathways, specially the glyoxylate and ribose phosphate metabolisms (FDR<0.05) for the tolerant sorghum under water deficit (Table 3).

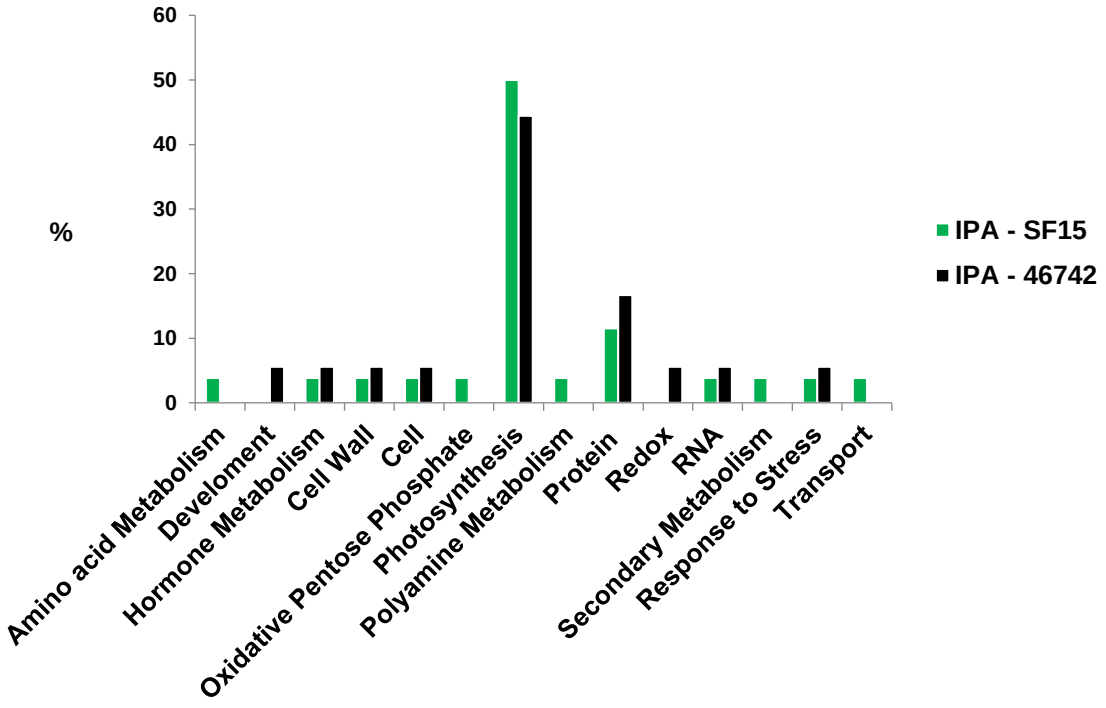


Figure 2. Biological process related to drought response in tolerant and sensitive varieties, after 72 h of water deficit.

Table 3. Enrichment analysis based in total differentially accumulated proteins in IPA-SF15sorghum plants after 72 h of water withholding.

Pathway description	Count in gene set	False discovery Rate
Biological Process		
Reductive pentose-phosphate cycle	4	1.92e-06
Carbon fixation	4	2.80e-06
Photosynthesis	5	2.02e-04
Ribose phosphate metabolic process	4	1.08e-02
Single-organism biosynthetic process	7	1.24e-02
KEGG Pathways		
Photosynthesis	3	1.19e-03
Carbon fixation in photosynthetic organisms	3	1.19e-03
Metabolic pathways	7	5.44e-03
Carbon metabolism	3	1.59e-02
Glyoxylate and dicarboxylate metabolism	2	1.77e-02
Microbial metabolism in diverse environments	3	4.06e-02

Table 4. Proteins more accumulated or exclusive of IPA-SF15sorghum plants after 72h of water withholding.

Biological Process	Spot	Ratio	ANOVA	Protein	ID	Theo. Mw/pI	Exp. Mw/pI	Organism	Cell. localization
Photosynthesis									
	201	**	0.01165	ATP synthase subunit alpha, chloroplastic	A1E9S1	55713/5.87	62024/5.57	*Sorghum bicolor*	Chloroplast
	111	↑7.87 %	0.03559	ATP synthase subunit beta, chloroplastic	A1E9T1	54036/5.31	53922/5.16	*Sorghum bicolor*	Chloroplast
	112	↑21.00 %	0.02287	ATP synthase subunit beta, chloroplastic	A1E9T1	54036/5.31	55030/5.07	*Sorghum bicolor*	Chloroplast
	113	↑10.41 %	0.04749	ATP synthase subunit beta, chloroplastic	A1E9T1	54036/5.31	53680/5.25	*Sorghum bicolor*	Chloroplast
	114	↑40.65 %	0.04467	ATP synthase subunit beta, chloroplastic	A1E9T1	54036/5.31	55842/5.00	*Sorghum bicolor*	Chloroplast
	6	↑31.27 %	0.05082	ATP synthase subunit beta, chloroplastic	A1E9T1	54036/5.31	56160/4.92	*Sorghum bicolor*	Chloroplast
	67	↑62.00 %	0.00483	Ferredoxin--NADP reductase	C5Z269	40279/8.31	35791/5.90	*Sorghum bicolor*	Chloroplast
	206	**	0.02284	Phosphoglycerate kinase	C5Z0B5	50190/5.89	50516/4.57	*Sorghum bicolor*	Chloroplast
	192	**	0.00291	Probable ribose-5-phosphate isomerase 3, chloroplastic	A0A1B6Q9A0	32578/6.62	30228/4.34	*Sorghum bicolor*	Chloroplast
	193	**	0.02859	Ribulose bisphosphate carboxylase large chain (Fragment)	A1E9T2	53323/6.33	55456/7.14	*Sorghum bicolor*	Chloroplast
	198	**	0.00545	Ribulose bisphosphate carboxylase large chain	A1E9T2	53323/6.33	57016/3.04	*Sorghum bicolor*	Chloroplast
	200	**	0.00024	Ribulose bisphosphate carboxylase large chain	A1E9T2	53323/6.33	55205/5.93	*Sorghum bicolor*	Chloroplast
	8	↓83.11 %	0.03709	Ribulose bisphosphate carboxylase small chain	E3WDK7	19366/8.77	15081/4.78	*Sorghum bicolor*	Chloroplast
Hormone metabolism									
	21	↑61.74 %	0.02539	ABA responsive element binding factor 1	C5XSV4	37698/9.32	20418/3.99	*Sorghum bicolor*	Nucleus
Protein metabolism									
	129	↑169.15%	0.01402	Hypersensitive to high light 1 chloroplastic	A0A194YRV2	28680/9.89	63979/6.21	*Sorghum bicolor*	Chloroplast
	197	**	0.01007	Serine/threonine-protein kinase PBL15	B4FVJ4	39445/9.74	54310/3.05	*Zea mays*	Cell membrane
	35	↓18.16 %	0.05305	Ubiquitinyl hydrolase 1	A0A1B6PIP5	88396/5.27	23450/5.45	*Sorghum bicolor*	Nucleus
RNA									

Table 4. Proteins more accumulated or exclusive of IPA-SF15 sorghum plants after 72h of water withholding (cont.)

	130	↑20.25 %	0.06619	Pentatricopeptide repeat-containing protein, mitochondrial	C5Y9D8	46135/6.07	64273/6.31	*Sorghum bicolor*	Mitochondrion
Transport									
	185	**	0.016239	ABC transporter B family member 19	A0A1B6PJF4	166145/8.88	24514/4.55	*Sorghum bicolor*	Plasma membrane/ Vacuole
Amino Acid Metabolism									
	189	**	0.00428	Arogenate dehydratase	A0A059PZM8	45927/6.73	45100/4.99	*Saccharum hybrid*	Chloroplast
Secondary Metabolism									
	191	**	0.00073	Flavin-containing monooxygenase	A0A1B6QR65	44209/5.35	66291/5.87	*Sorghum bicolor*	Cytoplasm
OPP									
	192	**	0.00291	Probable ribose-5-phosphate isomerase 3, chloroplastic	A0A1B6Q9A0	32578/6.62	30228/4.34	*Sorghum bicolor*	Chloroplast
Polyamine Metabolism									
	202	**	0.00001	Polyamine oxidase-like	A0A1B6QPW6	57205/5.44	61700/5.27	*Sorghum bicolor*	Endoplasmic reticulum
Response to Stress									
	207	**	0.00059	Calcium permeable stress-gated cation channel 1 isoform X1	C5YX25	88229/8.84	39374/5.94	*Sorghum bicolor*	Plasma membrane
Cell Wall									
	3	↑28.00 %	0.02871	Uncharacterized subfamily of plant invertase/pectinmethylesterase inhibitor domains	C5YBA9	23156/10.66	62734/5.16	*Sorghum bicolor*	Extracellular
Cell									
	127	↓39.00 %	0.01874	Fascin like domain (Actin crosslink)	C5Z4S2	61575/8.79	62777/5.50	*Sorghum bicolor*	Cytoplasm

** Exclusive of IPA-SF15; ID – Identifier from Uniprot; Mw - molecular weight;pI – Isoelectrical point; Theo.- Theorethical; Exp. – Experimental.

Table 5. Proteins more accumulated or exclusive of IPA-46742 sorghum plants after 72 h of water withholding.

Biological Process	Spot	Ratio	ANOVA	Protein	ID	Theo. Mw/ pI	Exp. Mw/ pI	Organism	Cell. localization
Photosynthesis									
	111	↓8.00 %	0.03559	ATP synthasesubunit beta, chloroplastic	A1E9T1	54036/5.31	53922/5.16	*Sorghum bicolor*	Chloroplast
	112	↓22.00 %	0.02287	ATP synthasesubunit beta, chloroplastic	A1E9T1	54036/5.31	55030/5.07	*Sorghum bicolor*	Chloroplast
	113	↓10.00 %	0.04749	ATP synthasesubunit beta, chloroplastic	A1E9T1	54036/5.31	53680/5.25	*Sorghum bicolor*	Chloroplast
	114	↓41.00 %	0.04467	ATP synthasesubunit beta, chloroplastic	A1E9T1	54036/5.31	55842/5.00	*Sorghum bicolor*	Chloroplast
	6	↓31.00 %	0.05082	ATP synthasesubunit beta, chloroplastic	A1E9T1	54036/5.31	56160/4.92	*Sorghum bicolor*	Chloroplast
	67	↓62.00 %	0.00483	Ferredoxin++NADPReductase	C5Z269	40279/8.31	35791/5.90	*Sorghum bicolor*	Chloroplast
	145	*	0.07003	Ribulose bisphosphatecarboxylase small chain	E3WDK7	19366/8.77	14475/5.52	*Sorghum bicolor*	Chloroplast
	8	↑83.00 %	0.03709	Ribulose bisphosphatecarboxylase small chain	E3WDK7	19366/8.77	15081/4.78	*Sorghum bicolor*	Chloroplast
Hormone metabolism									
	21	↓62.00 %	0.02539	ABA responsiveelement binding factor 1	C5XSV4	37698/9.32	20418/3.99	*Sorghum bicolor*	*Nucleus*
Protein metabolism									
	179	**	0.00011	F-box/kelch-repeat protein	C5Y7S1	41373/8.4	20804/5.59	*Sorghum bicolor*	Cytoplasm
	129	↓169.00 %	0.01402	Hypersensitiveto high light 1 chloroplastic	A0A194YRV2	28680/9.89	63979/6.21	*Sorghum bicolor*	Chloroplast
	35	↑18.16 %	0.05305	Ubiquitinyl hydrolase 1	A0A1B6PIP5	88396/5.27	23450/5.45	*Sorghum bicolor*	Nucleus
RNA									
	130	↓20.00 %	0.06619	Pentatricopeptide repeat containing protein,mitochondrial	C5Y9D8	46135/6.07	64273/6.31	*Sorghum bicolor*	Mithocondrion
Response to stress									
	176	**	0.00156	Protein disulfideisomerase-like 2-2 / Tioredoxin	C5XJT8	40447/6	28183/4.91	*Sorghum bicolor*	Endoplasmic reticulum
Redox									
	176	**	0.00156	Protein disulfideisomerase-like 2-2 / Tioredoxin	C5XJT8	40447/6	28183/4.91	*Sorghum bicolor*	Endoplasmic reticulum
Development									

Table 5. Proteins more accumulated or exclusive of IPA-46742 sorghum plants after 72 h of water withholding (cont)

	177	**	0.00027	E3 ubiquitin-protein ligaseCOP1 isoform X1	A0A194YSG7	78740/7.51	27441/4.90	*Sorghum bicolor*	Nucleus
Cell Wall									
	3	↓28.00 %	0.02871	Uncharacterized subfamily of plant invertase/pectin methylesterase inhibitor domains	C5YBA9	23156/10.66	62734/5.16	*Sorghum bicolor*	Extracellular
Cell									
	127	↑39.3 %	0.01874	Fascin like domain (Actin crosslink)	C5Z4S2	61575/8.79	62777/5.50	*Sorghum bicolor*	Cytoplasm

** Exclusive of IPA-46742; ID – Identifier from Uniprot; Mw - molecular weight; pI – Isoelectrical point; Theo.- Theorethical; Exp. – Experimental.

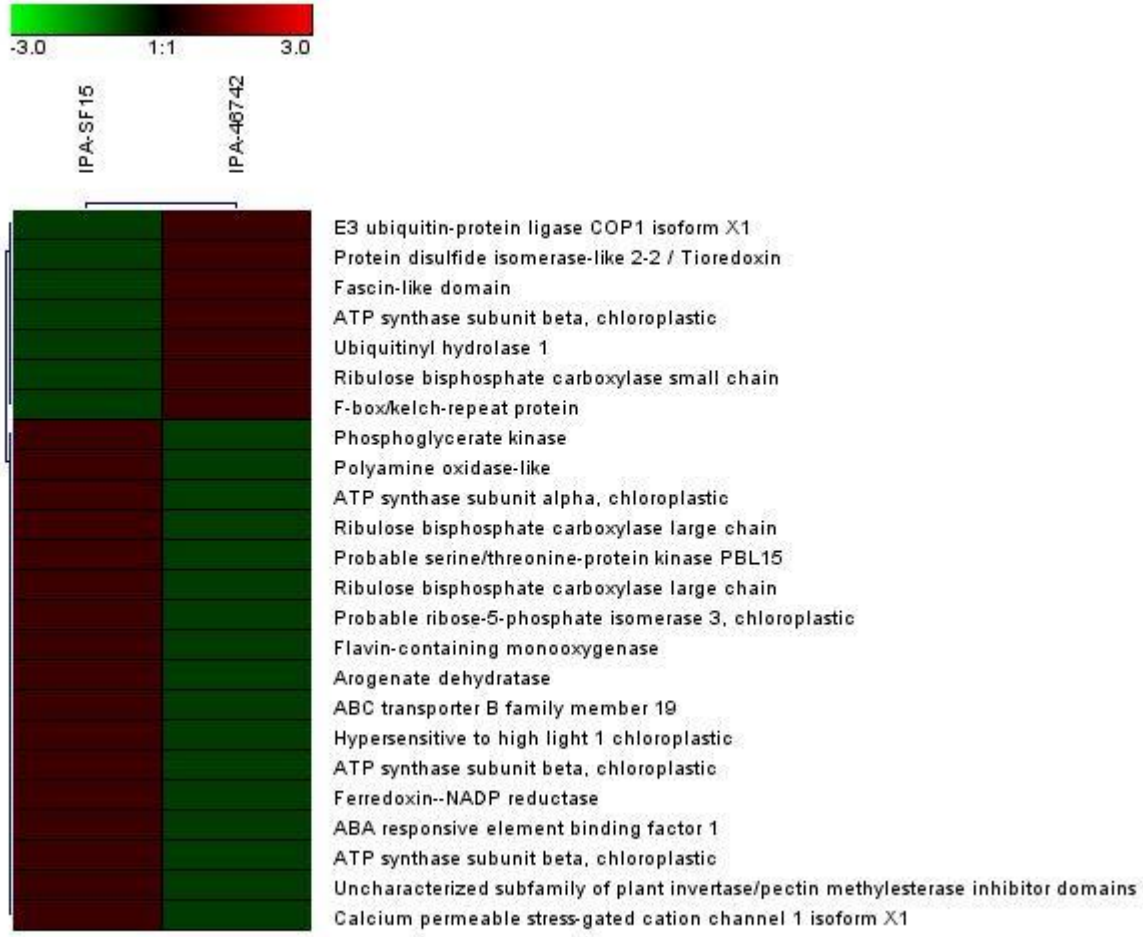


Figure 3. Hierarchical clustering of accumulation of total identified DAPs from the tolerant and sensitive sorghum varieties, based in Euclidean distance test.

4. Discussion

4.1 Physiological analysis

Just the water treatments had effect on RWC suggesting similar effects of drought in the tested varieties. However, data from photosynthesis and biochemical analysis showed that even with similar amounts of water, the IPA-SF15 and IPA-46742 have different responses toward water deficit. It seems that IPA-SF15 had better photosynthetic performance due to its capability to recover complete *Pn* after rewatering. Indeed, recovery of *Pn* is a good indicative of tolerance and might be related to the maintenance of photosynthetic apparatus during drought (Chen et al. 2016). Additionally, IPA-SF15 showed high efficiency of APX detox system, being able to readily respond the ROS burst at maximum drought in contrast to IPA-46742 that just responded ROS burst at recovering. In water deficit condition, the high levels of ROS are deleterious for plants due to oxidation of cellular structures and biomolecules, such as DNA, proteins and membranes (Imlay, 2013). In this context, the late adjustment of IPA-46742 must be dangerous and represent a disadvantage to this variety. However, we did not determine the activity of other peroxidases which may help in ROS detoxification, that would allow us wide analyses and interpretation (Yadav et al. 2014). Interestingly, IPA-SF15 maintained the levels of MDA which might be associated with APX detox activity and mitigation of membrane degradation. As expected, IPA-46742 had high levels of membrane peroxidation at rewatering. Not surprisingly, the APX activity was higher at this point, possibly to try to mitigate the ongoing degradation. Again, such late response of IPA-46742 is a disadvantage and might be related to non-complete photosynthesis recovery after rewatering. Indeed, sensitive varieties showed higher levels of MDA and non-effective antioxidant systems in comparison with the tolerant ones (Chakraborty and Pradhan, 2012).

Taken together, the results clearly show differences between the two varieties under drought, showing that IPA-SF15 is more efficient to adjust vital processes, even with the similar amounts of water of IPA-46742. Thus, such scenario leads us to classify, at least in our experimental conditions, IPA-SF15 as tolerant and IPA-46742 as sensitive.

4.2 Proteomics of tolerant IPA-SF15 versus sensitive IPA-46742

4.2.1 Photosynthesis

Most of the DAPs presented in the contrast between IPA-SF15and IPA-46742 were from photosynthesis pathways. The tolerant variety accumulated more ATP synthases subunits

and the large chain of rubisco (Table 4, Figure 3) than the sensitive one. This might indicate the ability of IPA-SF15 to maintain important components for CO_2 fixation, and such result must be related to the complete *Pn* recovery after rewatering, which was not registered in other variety (Table 1). During drought, the photosynthesis processes are impaired by several factors, mainly the CO_2 concentration and diffusion. After stomatal closure, the levels of carbon dioxide within mesophylls decrease, diminishing the photosynthesis, characterizing the stomatal limitations. In addition, the so called non-stomatal limitations also occurs, and it is related to other constraints such as the degradation of cellular structures and proteins from carbon anabolism, probably due to the overheat and lower transpiration (Carmo-Silva et al. 2012).

Despite the levels of CO_2 within IPA-SF15 mesophyll cells, it seems that such variety can overcome the imposed stomatal limitation by increasing the translation of carbon anabolism related proteins. Similarly, Chmielewska et al. (2016) found decreased concentration of photosynthesis related proteins in a sensitive variety of *Hordeum vulgare* submitted to drought stress, a fact not observed for tolerant one. In a drought tolerant rice genotype, higher accumulation of rubisco large subunit and ATP synthase subunit beta was registered, corroborating our results. In addition, such behavior may be found in other non-grass plants such as *Malus domestica* (Zhou et al. 2015) and *Arachys hypogaea* (Katam et al. 2016) drought tolerant varieties.

IPA-SF15 accumulates more ATP synthases and ferredoxin-NADP reductase, key enzymes of photochemical reactions, guaranteeing the production of reductant intermediates. Not surprisingly, Calvin cycle enzymes rubisco large chain and phosphoglycerate kinase, and PEP carboxylase (the main enzyme of CO_2 concentration in C4 plants), were also more translated, suggesting a synchrony pattern for photosynthesis adjustment. The latter enzyme higher accumulation might be due to the probable lower stomatal conductance and carbon dioxide content within mesophyll cells. Similar results were found in sorghum seedlings under saline and drought stress (Jedmowiski et al. 2014; Ngara et al. 2012), indicating that our results point out an efficient mechanism towards drought response, taking into consideration the intrinsic tolerance of *S. bicolor* to abiotic stresses (Ngara and Ndimba, 2014). On the other hand, only the sensitive variety showed higher concentration of rubisco small subunit (RbcS). The RbcS gene is localized in the nuclear genome and its translation occurs in the cytosol. After that, rubisco small unit is translocated to chloroplasts to form the entire rubisco complex (hexadecamer protein) (Berry et al. 2016). In this context, two possible situations

may have been achieved in IPA-46742 plants: the higher accumulation might represent a late response to drought or a tentative to overcome the degradation of rubisco complex, because RbcS levels in the stroma can lead to the synthesis of RbcL proteins in order to achieve the stoichiometry of the rubisco complex (Niyogi et al. 2015)

Additionally, such aspects are particularly relevant because under harmful conditions it is fundamental to maintain the integrity of photosynthetic pathways. Beyond the obvious reason of carbon fixation, several metabolic intermediates are derived from such pathways, being employed in other drought-related adjustment processes such as secondary metabolites and osmolytes synthesis. This might be a potential advantage for IPA-SF15 in detrimental to IPA-46742.

4.2.2 Hormone metabolism and transcription

The tolerant variety over-accumulated an abscisic acid (ABA) responsive binding element, which is a basic leucine zipper transcription factor (bZIP) that mediates the responses to ABA hormone. Several studies have been reported the effects of ABA in plants under abiotic stresses, mainly drought and salinity mediated by ABA binding factors (Yoshida et al. 2014; Shinozaki et al. 2017). Such transcription factors regulate drought adjustments in ABA dependent ways, by attaching the promoter regions of genes containing cis-ABRE elements (Choi et al. 2000; Hossain et al. 2010). It has been reported that ABA responsive elements improve the drought tolerance (Zhao et al. 2016; Na and Metzger, 2017) either by its own action or by a crosstalking with other ABA-signaling systems such as nuclear factor Y (NFY) or WRKY factors (Banerjee and Roychoudhury, 2017). In addition, several genes of efficient response towards water deficit are regulated by bZIP transcription factors (TF) such as dehydrins and late embryogenesis proteins (LEA), among others (Narusaka et al. 2003; Bielsa et al. 2016).

Additionally, IPA-SF15 plants translated more a mitochondrial protein pentatricopeptide (PPR), which is essentially a RNA-binding molecule. It has been proposed that this group of proteins, which are very abundant in plant kingdom, may act in various post-transcriptional events, keeping the integrity of different types of RNA and also in its transcription, processing and translation (Zsigmond et al. 2012; Manna, 2015). It seems that such function is relevant for abiotic stress responses because some studies registering more accumulation of PPR proteins have been reported, such as for cotton (Deeba et al. 2012). Confirming such idea, the overexpression of mitochondrial PPR genes enhanced the salt tolerance of *A. thaliana* seedlings (Zsigmond et al. 2012). To regulate the half-

life of RNA molecules is presumably essential for the maintenance of homeostasis, especially in an adverse environment. Abiotic stresses may degrade the cellular components, directly by heat or UV radiation, or even indirectly by the production of toxic compounds within plant tissues, which might oxidize DNA or RNA molecules. In this context, the PPR multieffects on RNA represents an advantage for IPA-SF15 sorghum, probably by mitochondrial homeostasis adjustments. However, the specific mechanisms of such proteins in order to alleviate the harmful effects of abiotic stresses remain unknown, despite the diversity of phenotypes potentially associated with PPR (Barkan and Small, 2014).

4.2.3 Protein Metabolism

This section represents the second more abundant category of total DAPs identified. Among them, an interesting hypersensitive to high light 1 (HHL1) protein was much more accumulated in tolerant sorghum. HHL1 is a thylakoid-binding protein, and it is an important component of photosystems repair and protein folding, being highly responsive to destructive effects of high light intensities (Jin et al. 2014). However, such protein is poorly characterized and studied in experimental conditions, existing just one curated accession in the entire Uniprot database. *A. thaliana* mutants of HHL 1 showed impairment on the photosystems when exposed to high light intensity, increased activation of xanthophyll cycle and high levels of reactive oxygen species (Jin et al. 2014). Taken together, such results pointed out the relevance of HHL1 in photosystem apparatus under harmful conditions. Interestingly, our sensitive variety had lower abundance of HHL1 and showed the highest levels of MDA, which might be related to lipid membrane oxidation, such as the thylakoids ones. Under drought conditions, several deleterious compounds may degrade cellular components (Imlay, 2013) and it is well accepted that drought and luminous stress occur simultaneously in dry environments, as like in our experiment. In addition, the sensitive sorghum has no efficient APX detoxifying ROS system which might be worsening the photosystems degradation.

Not surprisingly, tolerant sorghum also had more proteins from Calvin cycle, which reinforces the efficient maintenance of photosynthetic apparatus, suggesting a suitable strategy to deal with water deficit, mainly by photosystems disassembling/assembling HHL1 mediator, which seems to be absent or, more likely, in very low activity in the sensitive sorghum. As far as we know, this is the first report of HHL1 protein in a study

on water deficit, corroborating Jin and colleagues 2014 findings over the role of such protein in response to deleterious conditions to photosynthesis in land plants.

An ubiquitin hydrolase 1 (UBL1) was more translated in sensitive variety. UBL1 may act on the processing of ubiquitin-conjugated, providing monomers of ubiquitin to other ubiquitin-ligases or conjugating the protein target to ubiquitin monomers by itself mediation (Yan et al. 2000). The ubiquitin-system is a group of proteins related to polypetides-targeted degradation, whose gene products are commonly responsive to abiotic stress conditions, acting in protein turnover and replacement (Sharma et al. 2016). It is possible that the more accumulated UBL1 might reflect higher protein degradation, showing the difficulty for IPA-46742 to deal with water deficit.

More specifically, UBL1 may be working with another protein more accumulated in sensitive variety, the COP1 E3 ligase, which also act conjugating the degradation protein target and ubiquitin residue (Lyzenga and Stone, 2012). COP1 is known to regulate skotomorphogenesis/photomorphogenesis transition pathways (Josse and Halliday, 2008). In this context, it seems that in sensitive variety the skotomorphogenesis is being induced, which might be associated to the degradation of photosynthetic-related proteins, considering that heterotrophic events and substrates reallocation occur in skotomorphogenesis-induced plants (Humplik et al. 2017). Indeed, COP1 may play role in degradation of phytochromesand transcription factors that mediates the expression of photomorphogenesis related-proteins, such as HY5 and bZIPs (Xu et al. 2016, Hoecker, 2017), which may represent the inhability of IPA-46742 to cope with high light and drought damages. However, the mechanisms underlying such events have to be cleared.

In addition, serine/threonine kinase PBL15 was exclusive in tolerant variety. PBL15 belongs to a wide family of kinases which work in various cell processes through signaling cascades. It is located at plasma membrane, and several groups of serine/threonine kinases are known to regulate biotic response pathways (Afzal et al. 2008). PBL15 thrives responses to biotic stress, but in this work it was induced by drought. Some genes have similar *cis*-elements that might be regulated by different transcription factors in an event called cross-talking regulation (Agarwal et al. 2006). Literature reports higher accumulation of biotic stress related-proteins in experiments regarding abiotic stress factors (Katam et al. 2016; Cheng et al. 2018). For instance, simulation of drought conditions and treatment with ABA promoted higher abundance of leaf serine/threonine kinase (Td4IN2) transcripts, more than observed for cold or heat stress, in two *Triticum durum* seedlings varieties (Rampino et al. 2017). It is important to

stood out that physiological and molecular studies showed the usefulness of such varieties in wheat breeding programs for drought and other abiotic stress (Aprile et al. 2013).

4.2.4 Transport and response to stress

Water deficit may lead various harmful conditions to cell homeostasis, degrading proteins and also impair the correct protein folding during translation processes. For this reason, the plant cells have a set of foldase proteins which might help the correct folding of brand-new proteins (Ellgaard et al. 2018). Several studies have been reported the relevance of foldases in plants under water deficit, such as heat shock proteins (Park and Seo, 2015), peptidyl-prolyl-isomerases (Kaur et al. 2016), late abundant proteins - LEA (Hong-Bo et al. 2005) and peptide disulfide isomerases - PDI (Wilkinson and Gilbert, 2004). In this work, a PDI was exclusively accumulated in sensitive variety. PDI is located in the endoplasmic reticulum (ER) and it reduces cysteine (Cys) residues assuring the right fold of cys-rich-proteins (Wilkinson and Gilbert, 2004; Ellgaard et al. 2018), and its major accumulation in IPA-46742 suggests higher needs of chaperone activity due to drought deleterious effects. In this context, the first idea is to correlated PDI with an efficient response towards water deficit, however, PDI higher concentration may represent great amounts of unfolded proteins inside the ER and is probably related to ER stress event. Indeed, PDI accumulation and activity might be considered an indicative of ER stress (Lu and Christopher, 2008; Urade, 2009), especially when it takes into consideration the pattern of accumulation of other proteins found in the sensitive variety in this work.

Several proteins of photosystems and Calvin-cycle are regulated by thioredoxin system, acting on the reduction of S-S bonds, which may lead to target-protein activation, indicating that such proteins are Cys-rich (Niyogi et al. 2015). Besides the various Cys-rich target-proteins of PDI, we speculate that the sensitive variety is increasing the PDI-foldase system in order to overcome the degradation of photosynthetic protein apparatus, which it seems in agreement with skotomorphogenesis induction discussed here. On the other hand, ER stress may lead the activation of apoptotic pathways (Fu and Gao, 2014) and, if it is occurring, it represents another obstacle for IPA-46742 adjustment.

On the opposite side, the tolerant variety exclusively accumulated the plasma membrane transporters ATP binding cassette family B19 (ABCB19) and calcium stress-gate channel X1 (CSC1). ABC proteins belong to a wide family of compound transporters that act on the transit of different molecules such as secondary metabolites, lipid precursors and hormones, and ABCB19 isoform probably act on the auxin transport through the cells (Lin and Wang, 2005; Huang et al. 2016). Interestingly, Lin and Wang reported the role

of ABCB19 in auxin transport and the stimulation of photomorphogenesis in mutants of *A. thaliana*. Such mutants had impaired accumulation of chlorophylls and anthocyanins as well as the translation of RbcS, chlorophyll binding protein and photochlorophyllide reductase A. In agreement, Xin and Xue (2012) using LC-MS/MS proteomic tools, found increases on the accumulation of photosynthesis-related proteins in *A. thaliana* seedlings treated with auxin, reinforcing the role of such hormone in anabolic processes and photomorphogenesis.

Unlike ABCB19, the CSC1 is an ion channel of Ca^{2+} transport and may trigger cellular hyperosmotic shock responses (Hou et al. 2014). In these conditions, the influx of Ca^{2+} from apoplast increases the cytosolic Ca^{+2} levels, activating mechanisms to lead adjustments towards stress (Tang and Luan, 2017). CSC1 is an osmosensing and it seems to be determinant for drought or others osmotic-impaired conditions responses. *A. thaliana* mutants lacking the OCSC1 gene, a homologous of CSC1, showed impaired Ca^{2+} signaling in guard cells, and inefficient water adjustment, under osmotic stress promoted by sorbitol (Yuan et al. 2014). It is possible that IPA-SF15 perceives the water stress rapidly and transduces the Ca^{2+} signal more efficiently than IPA-46742, representing an advantage towards drought fit. This is particularly likely because Ca^{2+} signatures are specifically for stress stimuli and may lead to the appropriate response in order to maintain the cell homeostasis (Bose et al. 2011). Indeed, several tolerance adjustments are related to Ca^{2+} signatures such as ABA-dependent mechanisms (Jarzyniak and Jesinski, 2014).

4.2.5 Plant defense and secondary metabolism

Flavin-containing monooxygenase (FMO)is characterized by its function in oxidizing reactions, working in modifications of several molecules, by sulfoxidation, epoxidation and hydroxilation (Huijbers et al. 2014). In plants, it is associated with detoxification of deleterious compounds such as glycosinolates (Schlaich, 2007), and also participate on auxin biosynthesis as reported by Cha et al. (2015) in *A. thaliana* mutants overexpressing a flavin monooxygenase gene. Such mutants also showed drought tolerance and less ROS production. Higher accumulation of FMO in IPA-SF15 is consistent with aforementioned results however, inferences have to be made with parsimony, because FMO proteins belongs to different gene specialized families (Schlaich, 2007), and there is no characterization of such proteins in sorghum which has approximately 50 % of similarity with *A. thaliana* proteins. In addition, higher accumulation of FMO was found in C4 grass

Miscanthus sinensis under heat stress (Sharmin et al. 2013), reinforcing the role of such proteins in responses to abiotic stresses in grasses.

Additionally, tolerant variety translated exclusively an arogenate dehydratase that catalyzes the remotion of H_2O molecule from arogenate substrate, producing phenylalanine. Such amino acid is an important substrate for phenolics, reaction mediated by phenylalanine ammonia lyase (Pal). From this main pathway, several phenolics are synthesized such as cinnamic acids, monolignols, flavonoids, tannins and anthocyanins that are associated with responses to abiotic stresses (Vogt, 2010; Caliskan et al. 2017). Flavonoids may act in photoprotection and as antioxidant (Agati et al. 2013), cinnamic acids and monolignols are associated with lignin synthesis and consequent stiffening of cell wall in order to diminish water loss (Gall et al. 2015) and anthocyanins and tannins may play role in radicals scavenging with plant tissues (Cai et al. 2005). In this context, tannins are especially relevant because sorghum produces large amounts of tannins, manly the condensed ones (Nyachoti et al. 1997). Indeed, proteomics researches have been reported accumulation of proteins from secondary metabolisms pathways (Cheng et al. 2018), as well in sorghum under abiotic stresses. Ngara et al (2012) found higher concentration of proteins involved in cyanogenic glycosides metabolism. However, despite the potential role of secondary metabolism in drought adjustments, it is necessary metabolomic approaches to determine concentration of such molecules in our experiment to confirm such hypothesis.

4.2.6 Polyamine metabolism

Polyamines are cationic molecules with more than two amino groups, which may bind proteins, nucleotides and cellular structures, acting in maintenance of them, being important under stress (Rider et al. 2007; Rangan et al. 2014). The main plant polyamines are spermine, spermidine and putrescine and studies have been reported the positive outcomes of such molecules in plants under drought conditions (Wimalasekera et al. 2011). In our experiment, the tolerant variety accumulated a polyamine oxidase (PAO1) in an exclusive way. This enzyme plays important role in the polyamine catabolism, producing H_2O_2 and other products from a spermidine oxidation. In addition, PAO activity is important for polyamines back-conversion of spermidine to putrescine. Recent study showed that interconversion of spermidine to putrescine mediated by PAO occur in a C4 grass *Brachypodium distachyum* (Takahashi et al. 2017).

We did not quantify the PAO1 activity, but the higher accumulation of this enzyme in IPA-SF15 might indicate available spermidine for reaction, resulting in higher putrescine (Put) content. In agreement, Alcázar et al (2010) found drought tolerance improvement related to high levels of putrescine in *A. thaliana* transgenic lines showing improved Put biosynthesis. More interesting, the authors did not find increases in spermidine concentration which ratify the relevance of Put in drought conditions. However, for our results, it is necessary other analysis to confirm such idea.

Polyamines may contribute to abiotic stress tolerance beyond the osmotic adjustments. In such conditions, Put promote higher stability of thylakoid membrane, improving the light energy utilization and photosynthesis (Ioannidis and Kotzabasis, 2007; Kotakis et al. 2014). Such aspect might be related to the lower membrane lipid peroxidation of tolerant variety registered in our experiment. It is also consistent with accumulation of photosynthetic-related proteins and complete recovery of photosynthesis rate after rewatering found in IPA-SF15. For instance, Amooaghaie and Moghym (2011) found lower levels of MDA and electrolyte leakage in soybean seedlings treated with putrescine, and submitted to heat stress, corroborating our hypothesis. In addition, increases in polyamine concentration may lead the activation of several drought-responsive genes such as the ABA synthesis-related (Espasandin et al. 2014; Pál et al. 2018).

4.2.7 Cell wall

The osmotic changes within the cells during water deficit also lead structural changes in plasma membrane and cell wall, being one of the perception mechanisms of water absence and loss. Changes in cell wall structure and composition are important for diverse cellular processes such as cell elongation and defense against pathogens (Houston et al. 2016). Pectin methylesterases (PME) are enzymes involved in cell wall metabolism, more specifically on pectin. Such enzymes may trigger processes of both cell wall loosening or stiffening (Micheli, 2001) depending on the isoform, which may represent completely different outcomes to the cell. The probable inhibition of PME activity inferred by higher accumulation of PME inhibitor protein in IPA-SF15 sorghum variety might be related to cell wall stiffening, considering that loosening of cell wall occurs in cell elongation, processes undoubtedly impaired by water unavailability due to growth arrest (Tenhaken, 2015). In drought conditions, the lower availability of water diminish the necessary pressure to expand the cells, and thus inhibition of PME activity must be plausible as it appears to occur in IPA-SF15. Indeed, PME inhibitor is related to cell wall hardening as

found for in wheat (Hong et al. 2010), and in our experiment may be mediating processes of water loss mitigation.

4.3 What does the different accumulation in the contrasting sorghum varieties means?

Figure 3 shows a putative biological model of responses of the tested sorghum varieties under water deficit, based on the 2D proteomic analysis. Both proteomics and physiological data show clear differences between IPA-SF15 and IPA-46742, and in various aspects confirms each other. We considered IPA-SF15 as the tolerant variety and proteomics data shows intense improvement on photosynthesis, which is also supporting secondary metabolism. In addition, it seems that osmoregulation and osmoprotection also help the maintenance of cell structures, and the stress perception and signaling might permit rapid adjustments. It appears that the all aforementioned events are mediated by ABA, also including the role of auxins. Taken together, it is clear that IPA-SF15 employ diverse strategies to face water deficit which possibly helped it to reach metabolic recovery after rewatering.

In contrast, IPA-46742 did not show such efficiency, suffering much more under drought stress, and this is corroborated by higher induction of protein metabolism and ubiquitin pathways. In addition, IPA-46742 uses less of some desirable mechanisms to cope with drought, such as the maintenance of photosystems mediated by HHL1, however it is not clear if due to differences on patterns gene expression regulation between two varieties or due to direct damaging effects of stress itself. Opposite of tolerant variety, it seems that IPA-46742 activates heterotrophic processes such as the consuming of photosynthetic-related proteins, in apparently transition from photomorphogenesis to skotomorphogenesis. One possible explanation is the induction of endocycling processes which promotes a transient polyploidy and consequent increments in mRNA templates, forcing cell to respond the stress and also to grow (Humplik et al. 2017). In agreement with such idea, the ongoing ER stresses might lead PCD pathways induction. However, during stress such strategie is possibly to extreme and indicates the inability to deal with water deficit. Despite that the accumulation of skotomorphogenesis-related proteins indicate such possibility, it is still too speculative, needing complementary analysis such as karyotyping and other cytogenetics and microscopy approaches. In addition, it also necessary to quantify the levels of ABA hormone due to its role in triggering the aforementioned biological processes.

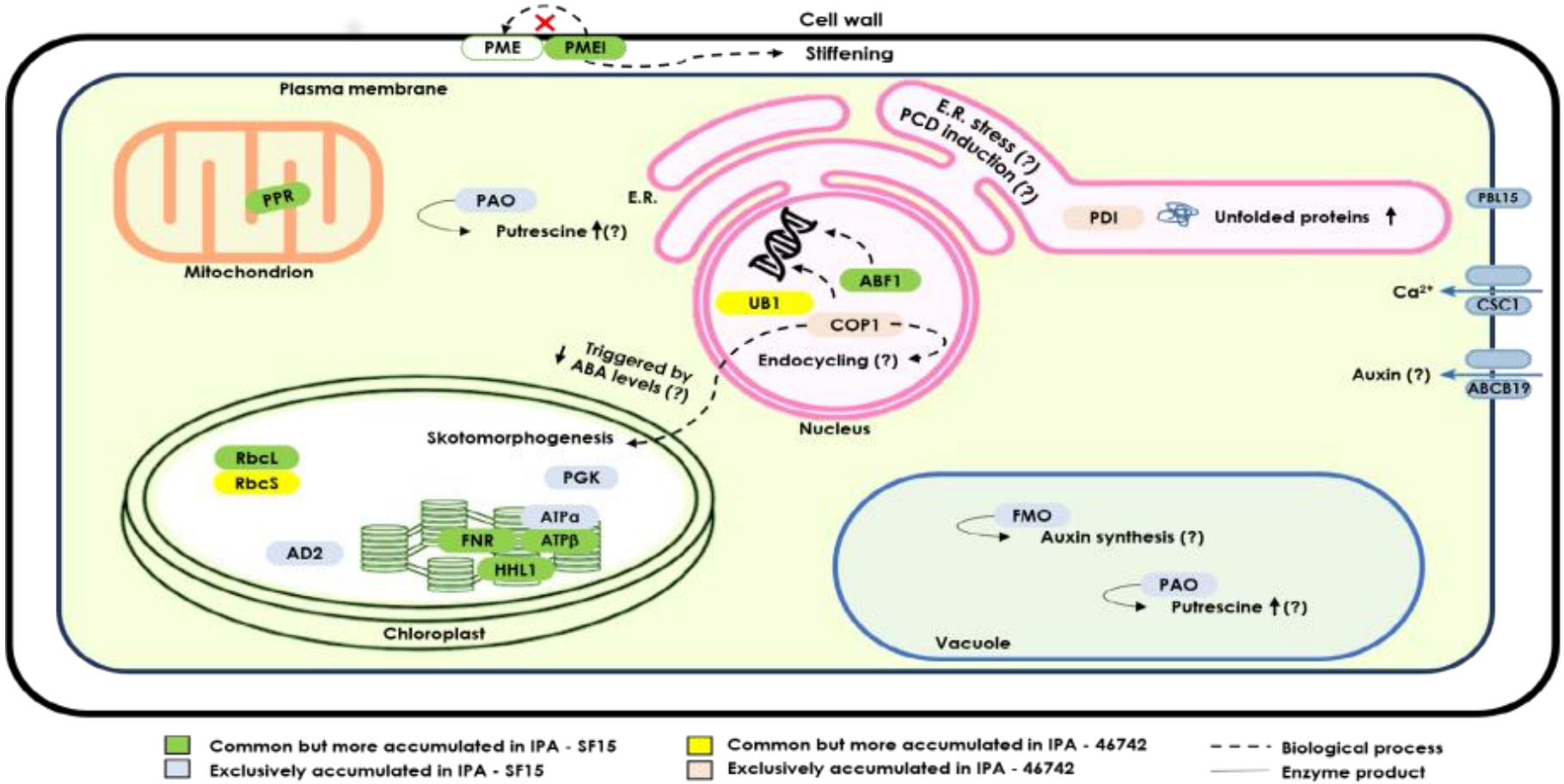


Figure 4. Putative biological model of proteomic response towards drought in IPA-SF15 and IPA-46742.

PAO- polyamine oxidase; FMO- Flavin-monoxygenase; ABC- ATP binding cassete transporter; CSC1 – Calcium stress-gated channel; PBL15 – serine/threonine kinase; ABF1- ABA binding factor 1; UB1 - Ubiquitin hydrolase 1; COP1 – Constitutive photomorphogenic 1; AD2- Aerogenate dehydratase; FNR – Ferrodoxin-reductase; HHL1- Hyper sensitive to high light 1; PGK – Phosphoglycerate kinase; ATP – ATP synthase; RbcL – rubisco large chain; RbcS – rubisco small chain; PPR- petatricopeptide; PDI – protein disulfide isomerase.

5. Conclusions

Shor-term drought stress impacts sorghum physiology and also proteomic response with evident differences between tolerant and sensitive variety. Taking IPA-SF15 as tolerance model, the integrity of photosynthesis-related structures and proteins is crucial for efficient maintenance of plant survival during drought and possibly complete recovery. In addition, the employment of diversified adjustment systems in different compartments of the cell is important, considering the wide side effects of water deficit. The lack or inefficient usage of such mechanism may be harmful, causing degradation of vital cellular structures and molecules, such as we found in IPA-46742. This is particularly relevant since sorghum is a drought tolerant crop and its pattern of responses might be useful in breeding programs of grass species. In addition, we suggest the HHL1 and COP1 as candidates for efficient and inefficient water deficit tolerance biomarkers, respectively.

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5 CONCLUSÕES

Em plantas, a manutenção da homeostase durante eventos de estresse ambiental é área de relevância biotecnológica, devido ao valor econômico de diversas espécies cultiváveis. Nesse contexto, os experimentos aqui realizados evidenciaram diferentes respostas frente ao déficit hídrico em três variedades de sorgo sacarino. A provável manutenção de estruturas membranares e do aparato fotossintético, mediada por um sistema antioxidante eficiente, apontam para mecanismos precoces de ajuste fisiológico, como observado em IPA-SF15 e EMBRAPA-BR506. Além disso, os dados proteômicos estão em aparente sincronia com os fisiológicos/bioquímicos, revelando as principais proteínas subjacentes ao fenótipo observado, pela metodologia empregada, indicando prováveis marcadores moleculares de resposta eficiente e ineficiente frente a escassez de agua.

Apesar de curto, o déficit hídrico imposto, conjuntamente às condições ambientais do experimento determinaram distintos fenótipos frente à seca, evidenciando que o ajuste precoce pode ser fundamental para o sucesso em tal ambiente hostil. Corroborando essa idéia, em condições de campo ou em estresses mais prolongados, variedades e genótipos tolerantes, de espécies gramíneas ou não, investem em mecanismos similares aos aqui encontrados. IPA-SF15 apresentou fenótipo tolerante sob condições de déficit hídrico, relacionado ao uso mais eficiente da água disponível na manutenção de processos fotossintéticos, mediado por diferentes mecanismos bioquímicos. Tais eventos tem respaldo nas principais proteínas mais acumuladas, mais notadamente àquelas de *turnover* de fotosistemas, ciclo de Calvin e síntese de osmólitos, indicando que a diversidade de mecanismos é importante para adaptação nas condições testadas.

Contrariamente, o fenótipo sensível observado em IPA-46742 pode se justificar pela resposta tardia às agressões oxidativas, possivelmente causando degradação de estruturas e moléculas, além de menor diversidade de alternativas bioquímicas para mitigar os efeitos deletérios. Dessa forma, se faz necessário o investimento em rotas de reparo de proteínas e realocação de nutrientes, como evidenciado nos dados proteômicos.

Os resultados aqui obtidos corroboram a relevância da caracterização fenotípica, por análises fisiológicas, atrelada a estudos de expressão gênica funcional, para compreensão mais completa de eventos biológicos de interesse acadêmico e econômico. Das diversas proteínas identificadas e responsivas ao déficit hídrico, pode-se sugerir a *hyper sensitive to high light 1* (HHL1) e *calcium stress-gated channel* (CSC1) como potenciais

marcadoras de resposta inicial e eficiente ao déficit hídrico, enquanto a E3 ligase-COP1 e *protein disulfide isomerase* (PDI) relacionadas à resposta ineficiente. Porém, estudos que elucidem a dinâmica de tradução sob diferentes regimes hídricos, bem como a validação de tais proteínas são necessários para corroborar tais hipóteses.

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