UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA
RENORBIO

RESPOSTA FISIOLÓGICA, BIOQUÍMICA, AGRONÔMICA E MOLECULAR EM AMENDOIM SUBMETIDO A DÉFICIT HÍDRICO

VALESKA SILVA LUCENA

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TESE DE DOUTORADO

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VALESKA SILVA LUCENA

Resposta fisiológica, bioquímica, agronômica e molecular em amendoim submetido a déficit hídrico

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LISTA DE ILUSTRAÇÕES

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

ABREVIAÇÕES E SÍMBOLOS

A: Fotossíntese líquida

ABA: Ácido abscísico

ACN: Acetonitrila

ERF8: “Ethelene Responsive Factors

APX: Ascorbato peroxidase

BAG: Banco ativo de germoplasma

Ca^{2+}: Cálcio

CAT: Catalase

C: controle

CHAPS: Ciclohexilamino dimetilamônio propano sulfonado

Ct: “*Cycle threshold”* limiar do ciclo

cm: Centímetro

CVA: Análise de variáveis canônicas

2D: Eletroforese bidimensional

Da: Dalton

Dae: Dias após a emergência

DPA: Dias após florescimento

Dpi: “Dots Per Inch” Pontos por polegada

DTT: Ditiotreitol

$D^{2:}$ Distancia de Mahalanobis

DREB: “Dehydration-responsive elemento binding”

E: Estresse

EiC: Eficiência instântanea da carboxilação

EUA: Eficiência de uso da água

gs: Condutância estomática

g: Gramas

GSH: Glutationa redutase reduzida

H_2O_2: Peróxido de hidrogênio

H_2O: Molécula de água

HC: Hábito de crescimento

HCCA: Ácido ciano-4-hidroxicinamínico

IAA: “Iodoacetamide” Iodoacetamida

IEF: focalização Isoelétrica

IRGA: analisador de gás com infravermelho

IPG: “Immobilized pH gel” Gradiente de pH Imobilizado

kDa: Quilodalton

L: Litro

mA: Miliamper

MALDI: “matrix assisted laser desorption ionization

MM: Massa molecular

mg: Micrograma Miligrama

Mr: Massa molecular relativa

MS: Espectrometria de massa

NADPH: Nicotinamida adenina dinucleotídeo reduzido

NCBI: “National Center for Biotechnology InformationNCED: 9’-cis-epoxicarotenóide dioxigenase

Ng: nanograma

NPK: Nitrogênio, fósforo e potássio

O_2: Oxigênio

$^{\cdot}OH$: Hidroxila

$O_2^{\cdot -}$: Ânion superóxido

$^1O_2$: Oxigênio singleto

P100S: Peso de 100 sementes

P100v: Peso de 100 vagens

PAGE: “Polyacrylamide Gel Electrophoresis

pIs: Potencial isoelétrico

PMF: “peptide mass fingerprint” Impressão digital de peptídeos

PMSF: fenilmetilsulfonilfluoreto

qPCR: Reação de polimerase em cadeia quantitativa

RNAm: Ácido ribonucleico mensageiro

ROS: Espécie reativa de oxigênio

RT: Transcriptase reversa

SDS: Duodecil Sulfato de Sódio

SOD: Superóxido dismutase

S/V: Número de sementes por vagem

TBP: Tributilfosfato

TB: Tipo botânico

TCA: Ácido tricloroacético

TCA: Ácido trifluorácetico

TEMED: N,N-Tetrametiletilenodiamina

TFA: Ácido trifluoracético

TOF: “Time of flight” Tempo de vôo

TS: Tamanho da semente

UPGMA “Unweighted Pair Group Method with Arithmetic Mean”

UV: Radiação ultravioleta

Vg/pl: Número de vagens por planta

VC: Variáveis canônicas

V: Volts

VP: Vinilpiridina

VCA: Análise de variáveis canônicas

W: Watts

RESUMO

As mudanças climáticas vêm afetando a produtividade de diversas culturas; dentre estas, a do amendoim (*Arachis hypogaea* L.) que se destaca pelo seu grande valor socioeconômico em regiões tropicais e sub-tropicais por ser a quinta oleaginosa mais cultivada no mundo. O estresse hídrico é um dos fatores abióticos que mais afetam sua produtividade. Embora as respostas ecofisiológicas desta espécie sejam estudadas, pouco se sabe sobre os eventos moleculares e suas relações metabólicas. Neste estudo inicialmente examinou-se a resposta fisiológica, bioquímica, agronômica, utilizando o método de variáveis canônicas (VCA) e UPGMA, em genótipos de amendoim submetidos a déficit hídrico, contribuindo na identificação de genótipos tolerantes a seca. A partir de experimentos com condições controladas as mudanças fisiológicas e da atividade das enzimas antioxidativas SOD, CAT e APX foram mensuradas na fase vegetativa de oito genótipos de amendoim submetidos a seis dias de suspensão hídrica total. A partir do qual as regas foram reestabelecidas e analisadas os danos agronômicos do estresse. Verificou-se que o método de UPGMA foi o mais contributivo na separação dos genótipos, destacando-se a linhagem CNPA 166 AM, precoce, como a mais promissora para trabalhos de melhoramento. Dentre as linhagens rasteiras a BR1 x LViPE-06(B) revelou comportamento moderado quando submetida ao déficit hídrico. Em nível proteômico foram identificados peptídeos diferencialmente expressos por 1DE e 2DE, envolvidos em diversas rotas metabólicas como fotossíntese, metabolismo energético, defesa antioxidativas e envolvidos na resposta ao déficit hídrico presentes em diferentes compartimentos celulares. Através do sequenciamento via MS dos peptídeos por 1DE foram identificados os peptídeos ATP sintase, peroxidase, taraxerol sintase e clatrina envolvidos na resposta ao dano sofrido no genótipo sensível LViPE-06 e peptidil-prolil-cis trans isomerase, beta-1,3-galactosiltransferase, fosfatase, citocromo p450 envolvidos no aumento da tolerância a seca em Senegal-55437. O genótipo tolerante Senegal-55437 foi escolhido para identificação das proteínas diferencialmente expressas relacionadas com tolerância a seca através do 2DE. Um total de 52 proteínas diferencialmente expressas foram encontradas, dentre as quais, 32 foram identificadas em tratamento com estresse hídrico e seis foram diretamente envolvidas em rotas de tolerância à seca. As proteínas estavam envolvidas na homeostase redox, na fotossíntese, na beta-oxidação e na osmoproteção. Sete proteínas foram encontradas, todas envolvidas em vários processos bióticos e abióticos, incluindo a tolerância à seca: kinesina, enoyl-CoA hidratase, subunidade do fotossistema I, fator de transcrição AP2 / ERF, helicase e proteína do tipo defensina. Os estudos pioneiros para estes genótipos de amendoim contribuíram para conhecer e avaliar as respostas adaptativas que culminaram em alterações metabólicas na identificação de genótipos promissores. Ao mesmo tempo, abre perspectivas para validação de novos marcadores que poderão ser utilizados em populações de melhoramento do amendoim visando à tolerância a seca e manutenção da produtividade de amendoim contribuindo para a estabilidade alimentar frente às mudanças climáticas e atendendo as demandas mundiais.

Palavras chaves: *Arachis hypogaea*; Estresse abiótico; Modulação fisiológica; Estresse oxidativo; Espectrometria de massa; Tolerância.

ABSTRACT

Climate changes are affecting the productivity of various crops; among these, the peanut (*Arachis hypogaea* L.) stands out for its great socio-economic value in tropical and sub-tropical regions to be the fifth most grown oilseed in the world. Water stress is one of the abiotic factors that affect productivity. Although the ecophysiological responses of this species are studied, little is known about the molecular events and their metabolic relationships. This study initially examined the physiological, biochemistry and agronomy response, using the method of canonical variables (VCA) and UPGMA in peanut genotypes subjected to water deficit, contributing to identify tolerant genotypes to drought. Physiological changes and activity of enzymes SOD, CAT and APX were measured in the vegetative stage eight peanut genotypes subjected to six days of total water suspension. From which waterings were re-established and analyzed the agronomic damage of stress. It was found that UPGMA method was more contributory to separate genotypes, especially the CNPA 166 AM early lineage, as the most promising for improvement work. Among the trailing lines to BR1xLViPE-06 (B) showed moderate behavior when subjected to water deficit. At the proteomic level, peptides differentially expressed by 1DE and 2DE, involved in several metabolic routes such as photosynthesis, energy metabolism, antioxidative defense and involved in the response to the water deficit present in different cell compartments were identified. Through the sequencing by MS of the peptides by 1DE the peptides ATP synthase, peroxidase, taraxerol synthase and clathrin involved in the response to the damage suffered in the sensitive genotype LViPE-06 and peptidyl-prolyl-cis trans isomerase, beta-1,3-galactosyltransferase , Phosphatase, cytochrome p450 involved in increased tolerance to drought in Senegal-55437. The tolerant genotype Senegal-55437 was chosen for identification of differentially expressed proteins related to drought tolerance through 2DE. A total of 52 differentially expressed proteins were found, of which 32 were identified in treatment with water stress and six were directly involved in drought tolerance routes. Proteins were involved in redox homeostasis, photosynthesis, beta-oxidation and osmoprotection. Seven proteins were found, all involved in several biotic and abiotic processes, including drought tolerance: kinesin, enoyl-CoA hydratase, photosystem I subunit, AP2 / ERF transcription factor, helicase and defensin-like protein. Pioneering studies for these peanut genotypes contributed to the knowledge and evaluation of the adaptive responses that led to metabolic changes in the identification of promising genotypes. At the same time, it opens perspectives for the validation of new markers that can be used in peanut breeding populations aiming at drought tolerance and maintenance of peanut productivity contributing to food stability in the face of climate change and meeting global demands.

Keywords: *Arachis hypogaea*; Abiotic stress; Physiological modulation; Oxidative stress; Mass spectrometry; Tolerance.

SUMÁRIO

CAPÍTULO I

1 Revisão de literatura

1.1 A cultura do Amendoim sob o aspecto socioeconômico

O mundo tem passado por uma série de mudanças tanto econômicas, quanto climáticas e enfrentado crises hídricas, que têm contribuído para elevação dos preços de commodities agrícolas, devido ao crescimento dos custos de produção e das despesas com o controle de qualidade que contribuem para volatilidade dos preços. Associado a isto, existe a crescente necessidade de produção alimentícia, devido ao crescimento dinâmico da população e consequentemente da demanda. Portanto, conhecer novas alternativas agrícolas, bem como adaptar as práticas de manejo, buscando-se aumentar a produtividade, minimizando a expansão e os danos ambientais constitui um dos principais desafios para a produção de alimentos. Como o ganho genético para produtividade tem sido baixo a cada ano, grandes esforços estão sendo direcionados pelos melhoristas no sentido de elucidar o potencial genético desta espécie, bem como, identificar cultivares mais adaptadas, visando o aumento da produtividade para cada região (FAO, 2015).

O amendoim (*Arachis hypogaea* L.) é uma *commodity* de grande importância mundial, cujos principais produtos no mercado internacional são os grãos consumidos *in natura* e o óleo. Seus grãos apresentam alto teor nutricional, cerca de 26% a 32% de proteínas e 40% a 55% de lipídios, especialmente ácidos graxos poli-insaturados como ômega 3 e ômega 6, indispensáveis ao metabolismo humano, vitaminas do complexo B (tiamina, riboflavina, niacina e vitamina E), além de resveratrol encontrado na película dos grãos, um potente antioxidante, e sais minerais (FREIRE et al., 2013; KRISHNA et al., 2015). É cultivado em mais de 80 países, especialmente no continente Asiático, destacando-se China, Índia, Nigéria, Estados Unidos e Sudão, responsáveis por uma produção mundial em torno de 45 milhões de toneladas de grãos. Esta cultura adapta-se a diferentes condições de solo, precipitações pluviométricas e temperaturas, apresentando ampla plasticidade fenotípica, sendo necessário, portanto, exploração do seu pool gênico para trabalhos de hibridação (USDA, 2015; KRISHNA et al., 2015).

No Brasil, sua produção concentra-se na região Centro-Sul, principalmente nos Estados de São Paulo, Minas Gerais, Paraná e Rio Grande do Sul, cujo aumento de área plantada passou de 108,9 mil/ha em 2015 para 121,1 mil/ha em Junho de 2016 e vem contribuindo para impulsionar o aumento da produção de grãos, a qual resultou em 346,8 mil/t toneladas na safra 2014/2015 e está estimada em 410,2 mil/t até Junho de 2016. Na região Nordeste a produção da safra 2015/2016 foi de 3,7 mil/t, destacando-se o Estado da Bahia, que também tem uma plantação extensionista. Porém, nos outros Estados o plantio de amendoim é normalmente realizado por pequenos produtores através da agricultura familiar na região semiárida, que apresenta, geralmente, baixos índices pluviométricos e de fertilidade do solo, o que pode comprometer a produtividade. Mesmo inserido nesta realidade, no Estado da Paraíba verificou-se um incremento, cuja área plantada passou de 0,3 para 0,7 mil/ha com a produção crescendo de 0,2 mil/t para 0,6 mil/t, o que corresponde a uma variação de 200% (CONAB, 2016).

Aumentos na produtividade, especialmente para a região semiárida, vem sendo obtidos após o lançamento de cultivares adaptadas para esta região como a BR1 e BRS 151 L7, de porte ereto e ciclo precoce, bem como a cultivar Pérola Branca de porte rasteiro, adaptadas para a região Sudeste (MELO FILHO e SANTOS, 2010). Estas cultivares foram desenvolvidas a partir dos trabalhos de melhoramento na Embrapa (Empresa Brasileira de Pesquisa Agropecuária), cuja estratégia tem sido explorar variabilidade genética nesta espécie botânica.

Atualmente há mais de 3000 mil acessos de amendoim no banco ativo de germoplasma (BAG) na (EMBRAPA/ CENARGEN), destes, cerca de 300 estão disponíveis para estudos de pré-melhoramento na Embrapa Algodão existindo pouca informação sobre os efeitos desses acessos quando submetidos a estresse hídrico. Os progressos dos programas de melhoramento têm contribuindo para a estabilidade da produção, melhoria da qualidade dos frutos para diversificar sua utilização, desenvolvimento de cultivares com maior teor de óleo, ácido oleico, resistentes a doenças e de maior tolerância a estresse hídrico (MALLIKARJUNA e VARSHNEY, 2014).

Os fatores que mais afetam o crescimento e desenvolvimento desta cultura são a temperatura, nas fases de florescimento, crescimento e maturação dos frutos e o déficit hídrico especialmente na produção e enchimento das vagens com sensibilidade variada de acordo com o genótipo (SHINDE et al., 2010; NAUTIYAL et al., 2012). Tais situações causam alterações fisiológicas que culminam em modificações metabólicas

afetando seu crescimento e perdas na produtividade (KALARIYA et al., 2013). A diminuição destas perdas tem ocorrido devido a esforços na área de melhoramento associadas a aplicações biotecnológicas possibilitando a identificação de cultivares tolerantes atreladas a melhorias nas técnicas de manejo.

1.2 Caracterização da espécie *Arachis hypogaea* L.

O amendoim cultivado, cujo nome está relacionado com a palavra da língua tupi, *mandu'wi* e significa enterrado, é uma espécie herbácea pertencente à família Fabaceae, gênero *Arachis* e espécie *A. hypogaea*, com hábito de crescimento ereto ou rasteiro (Fig. 1A e 1B). Apresenta flores sésseis com coloração alaranjada ou amarelada que depois de fecundadas, se desenvolvem em uma estrutura chamada ginóforo de geotropismo positivo que propicia o desenvolvimento dos frutos em forma de vagens (Fig. 1C e 1D) dentro das quais ficam as sementes (Fig. 1E e 1F) (SANTOS et al., 2013).

Fig. 1 Hábito de crescimento ereto (A) e rasteiro (B), padrão das vagens (C) e (D) e das sementes da subsespécie *fastigiata* (E), com 3 a 4 sementes por vagem e de tegumento vermelho e *hypogaea* (F), com 1 a 2 sementes por vagem e de tegumento bege
Fonte. Roseane C. Santos.

Apresenta centro de origem incerto, podendo ter ocorrido no Peru, onde existem espécies diplóides (*A. duranensis* e *A. ipaensis*) ou no noroeste Argentino e região oriental dos Andes, onde existe uma espécie geneticamente muito similar (*A. monticola*). Provavelmente nestes locais deve ter ocorrido a hibridação dos ancestrais diplóides *A. duranensis* (BB) e *A. ipaensis* (AA), gerando as espécies tetraploides

(2n=2x=40, AABB). Tendo como centros naturais de dispersão: Brasil, Bolívia, Paraguai, Argentina e Uruguai (VALLS e SIMPSON, 2005; GRABIELE et al., 2012). Existem ainda várias outras espécies diplóides selvagens com diferentes genomas (A, B, D, F e K) (SAMOLUK et al., 2015). Por ser uma espécie de grande importância econômica teve seu genoma sequenciado pela Iniciativa Internacional do Genoma do Amendoim (IPGI) que demonstrou grande conservação no conteúdo de genes com outras leguminosas (SHIRASAWA et al., 2013).

A espécie *A. hypogaea* está subdividida em duas subespécies: *hypogaea*, com o tipo botânico Virgínia (*Runner*), apresenta 1 a 2 sementes por vagem, porte rasteiro, tegumento bege, ciclo longo sem flores no eixo central e com ramificações alternadas entre ramos vegetativos e reprodutivos (Tabela 1); e a subespécie *fastigiata*, que apresenta as cultivares mais precoces e os acessos mais tolerantes a seca, com os tipos botânicos *Spanish* que contém 2 sementes por vagem, porte ereto, tegumento vermelho e alto teor de proteína e *Valência* com 3 ou 4 sementes por vagem, porte ereto, tegumento vermelho, teor variável de óleo e proteínas (Tabela 1). Existe ainda a variedade hirsuta, que ocorre no Peru, de ciclos mais longos, maior pilosidade nos folíolos, porte rasteiro e apresenta ainda como principais atributos econômicos alto teor de óleo e produtividade. Apesar da maior capacidade para produção de grãos dos genótipos rasteiros, estes exigem técnicas de manejo mais intensivas, o que reflete no custo de produção superior ao dos tipos eretos (NOGUEIRA et al., 2013).

Tab 1 Características descritivas de diferentes tipos de amendoim

Tipo Agrícola	**Porte**	**Hábito**	**Nº S/V**	**CT**
Virgínia	Rasteiro	Tardio (120-160 dias)	1 a 2	Bege
Spanish	Ereto	Precoce (85-110 dias)	2	Vermelho
Valência	Ereto	Precoce (85-110 dias)	3 a 4	Vermelho

S/V – sementes por vagem; CT – cor do tegumento. Fonte: Santos et al (2013)

A combinação destes materiais via cruzamentos pode agregar valores não apenas com relação à produtividade, mas também, de tolerância à seca, por isso, o aumento da demanda por cultivares de amendoim adaptadas para a região semiárida tem feito com que os programas de melhoramento busquem hibridação de genótipos do tipo *Valência* (eretos e precoces) com os de tipo botânico *Runner* (rasteiros e tardios). Tais

cruzamentos buscam desenvolver cultivares que possam responder estavelmente no aspecto de produção, mesmo quando submetidos a situações de baixa e irregular disponibilidade hídrica (SANTOS et al., 2013).

1.3 Estresse Ambiental e déficit hídrico

As perdas relacionadas com a seca têm sido o principal desafio para a produção de grãos, sendo este, o fator que mais limita a produtividade, especialmente na região semiárida. Diversos tipos de condições ambientais desfavoráveis podem fazer com que as plantas tenham dificuldade em absorver água (CHAKRABORTY et al., 2015).

O amendoim é frequentemente exposto a situações de estresse que afetam seu crescimento, desenvolvimento e produtividade. Estes estresses podem ser bióticos ou abióticos. Os principais danos abióticos, a que estão submetidos, são altas ou baixas temperaturas, excesso de salinidade no solo, nutrição mineral inadequada, exposição à excessiva radiação UV, excesso de metais no solo, excesso de água ou déficit hídrico, sendo este último mais comum na região semiárida devido à distribuição irregular de chuvas ou irrigações (BRAY et al., 2000)

A duração, severidade e proporção do estresse imposto, influenciam na resposta das plantas. Condições adversas severas podem iniciar respostas diferentes para um mesmo tipo de estresse. E essa resposta pode ser causada diretamente pelo estresse ou pode resultar do acúmulo de danos no metabolismo. Portanto, é importante elucidar como as plantas respondem a determinada situação de estresse para manutenção do seu crescimento e desenvolvimento.

Vários autores reportam sobre a plasticidade fenotípica e capacidade do amendoim de se adaptar a condições adversas como altas temperaturas e escassez hídrica, em graus variados dependendo da cultivar. Azevedo Neto et al (2009) ao avaliarem as respostas fisiológicas e bioquímicas, em amendoim submetidos a seca por 3, 5, 9, 12 16 e 20 dias, verificaram respostas similares em nível fisiológico aos 3 e 5 dias de estresse hídrico, porém observaram redução nestes parâmetros aos 9 dias de estresse, que se manteve em declínio pelos períodos subsequentes. Considerando que a tolerância ao estresse hídrico é um evento complexo, a identificação de genótipos tolerantes e produtivos requer estudos em diversas áreas visando conhecer suas adaptações e respostas sob condições específicas. Como o estresse prolongado incide sobre a produtividade agrícola, conhecer e prevenir estes danos tem sido metas nos

programas de melhoramento desta cultura e como a expressão gênica ocorre antes que o fenótipo se torne observado, análises entre os períodos de 5 a 9 dias devem ser consideradas visando à identificação das respostas em nível molecular.

Algumas plantas têm tolerância ao déficit hídrico porque suas características fisiológicas, bioquímicas e morfológicas facilitam a tolerância a ambientes secos. No âmbito fisiológico o fechamento estomático tem sido uma das primeiras alterações verificadas no amendoim que contribui para sua estabilidade e principalmente pela redução da transpiração. (TAIZ e ZIEGER, 2004). Em nível bioquímico as alterações mais evidentes tem sido o aumento de solutos compatíveis e de enzimas do complexo antioxidativo buscando minimizar o acúmulo de ROS (espécies reativas de oxigênio). Ao mesmo tempo, ocorrem alterações morfológicas a médio e longo prazo, como espessamento da cutícula, redução da área foliar e aumento da produção de raízes. Estas mudanças podem ocorrer em resposta a expressão de genes que resultam da injúria ou dano que ocorre durante o estresse hídrico.

A regulação destes processos pode depender das condições ambientais. Os genes podem estar associados com alterações no metabolismo, regulação, sinalização, ou reconhecimento do estresse. De acordo com Bray et al (1993), por exemplo, a concentração de ABA é alterada em diferentes condições de seca o que estaria relacionado com diferenças no nível de expressão de genes envolvidos com este hormônio durante o estresse hídrico e que a regulação do transporte intracelular de ABA pode representar uma nova estratégia para a produção de cultivares de maior tolerância a seca.

1.3.1 Modificações fisiológicas, bioquímicas, morfológicas e agronômicas

As plantas respondem diferentemente ao déficit hídrico, onde a característica mais comum é a redução do turgor, indicando perda de água por transpiração e falha na reposição de água, o que pode afetar o crescimento e o desenvolvimento da planta. Conhecer as respostas fisiológicas durante o estresse hídrico é de fundamental importância para identificar alternativas que minimizem seus efeitos deletérios (CHAKRABORTY et al., 2015).

A deficiência hídrica induz uma série de mudanças fisiológicas com o intuito de manter a hidratação do tecido ou de aumentar a tolerância da planta, possibilitando seu funcionamento mesmo quando desidratada. Uma das primeiras alterações é o

fechamento estomático. As folhas contêm estômatos nas duas superfícies e sob condições normais as condutâncias foliares são equivalentes, porém, sob condições de estresse hídrico, os estômatos da superfície abaxial são mais sensíveis respondendo mais rapidamente as variações de água da planta (NOGUEIRA et al., 2013). Ainda em condições normais, a absorção de íons, estimulada pela luz, e o acúmulo de solutos reduzem o potencial osmótico das células, resultando na entrada de água nas células-guardas, traduzindo-se em abertura dos estômatos, porém, ao perderem estes solutos, ocorre perda de turgor, redução da condutância estomática para minimizar a perda de água, o que acaba inibindo a absorção de CO_2 indispensável para a fotossíntese (AMINIFAR et al., 2013).

Além disso, o excesso de energia luminosa pode danificar especialmente o fotossistema I (P700) levando ao acúmulo de NADPH e escape de elétrons que reagem com o oxigênio molecular levando ao acúmulo de espécies reativas de oxigênio (ROS), como o ânion superóxido $O^{2\bullet-}$, oxigênio singleto $^1O_2$, radical hidroxila $^{\bullet}OH$ ou espécies não radicais como o peróxido de hidrogênio H_2O_2. Estes ROS são tóxicos para o metabolismo celular, ocasionando a peroxidação dos lipídios, inativação de enzimas e degradação do DNA culminando na morte celular. Assim um sistema antioxidativo eficiente mantém a homeostasia da célula mesmo em condições de estresse hídrico (BARBOSA et al., 2014).

A formação do oxigênio singleto está relacionada com a super excitação da clorofila, que pode danificar a proteína D1 do fotossistema II, que necessita de tempo hábil para ser produzida novamente, período no qual a clorofila triplet transfere elétrons para o oxigênio molecular formando o $^1O_2$, que é altamente danoso especialmente para os cloroplastos.

O ânion superóxido pode ser produzido por sucessivas adições de elétrons ao O_2, ou por alterações fisiológicas devido ao fechamento estomático e redução da fotossíntese levando a diminuição da oxidação do NADPH, devido ao forte poder redutor da ferredoxina que deveria passar elétrons para o NADP e o transfere para o O_2 formando $O^{2\bullet-}$. Este pode atuar em reações de oxidação e redução para formar o radical hidroxila ($^{\bullet}OH$), o mais reativo de todos os radicais, mas está presente apenas em pequena proporção em pH fisiológico, enquanto o H_2O_2 pode ser formado através do aumento da fotorrespiração nos peroxissomos ou através da reação com o superóxido (SHARMA et al., 2012).

Uma vez que estes radicais aumentam na célula, podem ser convertidos através de mecanismos de defesa não enzimáticos envolvendo a ação do ascorbato, glutationa redutase, prolina, como também os carotenóides, presentes em grande quantidade na película dos amendoins, que atuam como fotoprotetores do aparelho fotossintético, absorvendo a energia excessiva, impedindo a reação do oxigênio molecular para formar o oxigênio singleto ($^1O_2$) ou converte-o para uma forma menos ativa. Os meios enzimáticos envolvem comumente a ação das enzimas superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase (APX) (AKCAY et al., 2010).

A SOD tem sido relatada como a primeira linha de defesa, distribuída pelo citosol, cloroplastos, peroxissomos e mitocôndria. Catalisa reações de dismutação, em que átomos de ânion superóxido são oxidados e reduzidos a O_2 e H_2O_2, diminuindo o risco de formação do $^{\bullet}OH$ a partir do $O_2^{\bullet-}$. Uma vez formado, se em baixas concentrações, o peróxido pode atuar na defesa contra estresse biótico, participando de reações de oxidação de aminoácidos da parede celular vegetal alterando sua estrutura possibilitando maior resistência contra insetos. Porém, em altas concentrações o peróxido oxida cistinas e metioninas o que pode inativar enzimas do Ciclo de Calvin indispensáveis para o processo de fixação do CO_2 e produção de carboidratos (TAIZ e ZEIGER, 2004).

A CAT, opera sem agente redutor, fornece às plantas uma forma energeticamente eficiente, principalmente em concentrações relativamente altas de H_2O_2, atua dentro dos peroxissomos e glioxissomos formando O_2 e H_2O. A APX é considerada a enzima mais importante da eliminação de H_2O_2 no citosol e nos cloroplastos e tem sido eficiente em alguns compartimentos celulares onde não existe catalase. Porém, a APX necessita de um doador de elétrons, geralmente o ascorbato, e por isso atua conjuntamente com o antioxidante não enzimático glutationa redutase (GSH) na eliminação do excesso de ROS (BARBOSA et al., 2014).

Associadas as estratégias de sobrevivência, buscando-se o ajustamento osmótico e a minimização dos danos oxidativos, ocorrem alterações morfológicas como maior enrolamento ou maior inclinação das folhas, diminuindo ainda mais a absorção de luz e consequentemente à fotossíntese. Assimilados são alocados para órgãos reprodutivos como estratégia de sobrevivência e para as raízes, provocando inibição do crescimento da parte aérea; ainda pode ocorrer redução do ciclo, propiciando a reprodução e perpetuação da espécie antes que o déficit hídrico se torne muito severo (CAVATE et al., 2011).

Estas modificações metabólicas culminam em alterações agronômicas tais como redução na produção das vagens e no peso das sementes, uma vez que metabólitos estão sendo alocados para manter o metabolismo da planta, diante das condições extremas, refletindo diretamente na produtividade desta cultura.

1.3.2 Aspectos moleculares em resposta ao déficit hídrico

O estresse é responsável por iniciar uma série de modificações em nível celular que podem ser monitoradas através das ômicas. O conhecimento sobre rotas metabólicas e suas modificações em resposta ao estresse hídrico pode ser compreendida através de análises de expressão gênica por técnicas moleculares como RT-PCR, qRT-PCR, bibliotecas subtrativas, microarranjos, entre outras, que têm fornecido informações importantes de genes diferencialmente expressos em determinada variedade ou tecido em resposta ao déficit hídrico (ABAR, PLDa1, TOC1, DREB, LEA, dentre outros) (AHUJA et al., 2010). Isso possibilita a compreensão dos mecanismos genéticos de características agronômicas importantes para o melhoramento de amendoim. Entretanto, estas técnicas não refletem diretamente a expressão de proteínas, pois muitos transcritos podem sofrer modificações pós-transcricionais ou pós-traducionais que consequentemente podem interferir na conformação e função proteica, portanto as proteínas servem como ponte entre a informação codificada no genoma e o fenótipo (ZHU et al., 2013).

As proteínas produzidas a partir de genes induzidos pelo estresse podem se acumular em resposta a condições desfavoráveis, atuando para minimizar seus efeitos, levando ao aumento da tolerância do genótipo. Modificações na expressão gênica culminam em respostas fisiológicas que podem ser independentes do ABA (ácido abscísico), via ácido jasmônico e etileno, ou por vias dependentes do ABA, um metabólito secundário do grupo dos terpenos, que é produzido normalmente pelas plantas, mas é acumulado quando as plantas estão submetidas ao estresse hídrico (NAKASHIMA et al., 2015).

Acredita-se que o ABA sintetizado nas raízes atue como um sinalizador, promovendo o fechamento estomático e influenciando nos demais aspectos fisiológicos da resposta ao estresse. Nos plastídios, a síntese deste hormônio ocorre pela rota dos carotenoides e também está relacionado com o acúmulo de proteínas e lipídios de reserva durante o período de embriogênese de sementes (TAIZ e ZEIGER, 2004).

É durante a embriogênese que naturalmente ocorre redução do teor de água, levando ao aumento da síntese de NCED (9'-cis-epoxicarotenóide dioxigenase), enzima rapidamente induzida pelo estresse hídrico que catalisa a clivagem de precursores que se transformam em ABA, influenciando na resposta fisiológica da planta. O ABA citosólico também pode aumentar durante situações de estresse como resultado do aumento da sua síntese nas folhas, redistribuição pelas células do mesófilo, importação das raízes e movimentação a partir de outras folhas. Por isso, o monitoramento do aumento da expressão de genes em resposta ao déficit hídrico tem sido utilizado como um potencial marcador, demonstrando a interação molecular e sua complexidade na resposta fisiológica, relacionado com a redução do potencial hídrico possibilitando a absorção de água do solo (LIU et al., 2014).

Através destas sinalizações ocorrem modificações metabólicas (Fig. 2) que culminam no acúmulo de solutos compatíveis, aumento da atividade de enzimas do complexo antioxidativo (SOD, CAT e APX), indução na expressão de proteínas hidrofílicas (LEA - *late embriogenesis abundant*), associadas à capacidade de reter água, evitando a desidratação e estabilização de membranas, ativação de proteases com o intuito de remover ou reciclar enzimas desnaturadas, expressão de proteínas de choque térmico, que atuam na proteção ou renaturação de proteínas inativadas por desidratação (TAIZ e ZEIGER, 2004).

As análises a nível transcricional têm ampliado a compreensão das respostas moleculares e da interação dos genótipos em determinado ambiente. A qRT-PCR (*Quantitative reverse transcription-polymerase chain reaction*) tem sido uma das técnicas preferidas para análise e quantificação de transcritos de baixo ou alto nível de expressão por ser uma técnica muito sensível e relativamente fácil (MORGANTE et al., 2011).

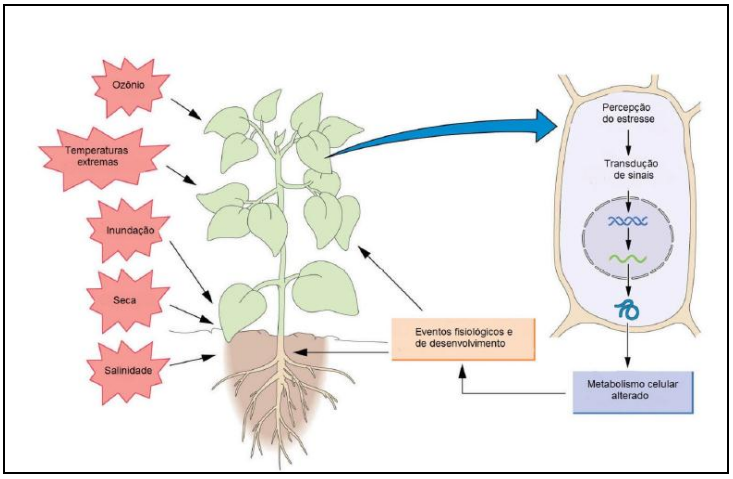

Fig. 2 Alterações na expressão gênica em resposta a condições ambientais desfavoráveis. Fonte: Bray et al (2000).

Esta técnica possibilita acompanhar a amplificação de genes pelo aumento da emissão de fluorescência a cada ciclo, sendo o sistema *Sybr Green*, um dos mais utilizados, pela ação do corante intercalante que se liga aos amplicons, emitindo a fluorescência. Devido a esta sensibilidade ela vem sendo utilizada para quantificar um transcrito em particular, quantificação absoluta ou medir a alteração na expressão de um gene específico, comparando-se com a expressão de genes de referência, quantificação relativa.

Os recentes avanços do método possibilitam a identificação do período que a reação atinge o limiar da fase exponencial ou Ct (*cycle threshold*) permitindo a quantificação exata e reprodutível dos produtos amplificados. Portanto, quanto menor a quantidade de moléculas maior o valor de Ct. A acurácia desta técnica é influenciada por diversas variáveis como integridade do RNA, eficiência enzimática na síntese do cDNA e amplificação por PCR (Reação de polimerase em cadeia) e atividade transcricional das células e tecidos analisados. Essas variáveis são geralmente controladas pela normalização da expressão gênica com genes de referência, cuja expressão é assumida como constitutivo (CHI et al., 2012).

Embora tenham sido identificados diversos avanços na área da transcriptômica, evidências sugerem que a função das proteínas de transcritos é influenciada por mecanismos regulatórios pós-traducionais que aumentam a quantidade de RNAm específico, amplificam sua tradução, estabilizam proteínas, alteram sua atividade ou ocorre a combinação de alguns destes. Assim, necessitam-se estudos associados de transcriptômica, proteômica e metabolômica para elucidar estes mecanismos.

1.4 Análise proteômica

A análise de proteômica hoje é tida como uma das ferramentas mais poderosas por possibilitar a compreensão sobre a regulação da expressão e o funcionamento do genoma, influenciados por alterações ambientais permitindo uma melhor compreensão dos eventos celulares que culminam em enorme impacto econômico, como a identificação qualitativa e quantitativa de genes presentes em uma determinada organela, célula, tecido, órgão ou estágio de desenvolvimento e que estejam se expressando em um estádio de desenvolvimento específico, ou sobre diferentes condições de estresse biótico ou abiótico. Desta forma, esta ferramenta permite identificar proteínas expressas durante o desenvolvimento de frutos (ZHU et al., 2013), contra danos bióticos, como inibidores de proteases (LI et al., 2013) ou relacionada com aspectos fisiológicos (FAGHANI et al., 2015).

Portanto, a exploração da variabilidade em nível ômicas tem possibilitado a identificação de genes, seu padrão de expressão e regulação possibilitando sua manipulação. A implementação de métodos mais rápidos e sensíveis para identificação de proteínas através de eletroforese unidimensional (1-DE) ou bidimensional (2-DE), combinada com cromatografia e espectrometria de massa (MS), têm se tornado uma técnica que possibilita a identificação temporal, bem como, diferenças nos níveis de expressão nos diferentes genótipos quando expostos a condições ambientais ou que apresentam características fisiológicas distintas (CÁNOVAS et al., 2004; FAGHANI et al., 2015).

A análise de proteínas em genótipos submetidos ao estresse hídrico tem revelado respostas diferenciais em genótipos contrastantes. Kottapali et al. (2009), por meio de eletroforese 1-DE e 2-DE associada a espectrometria de massa de vôo ionizante (MALDI-TOF). Estes mesmos autores identificaram 102 spots correspondentes a proteínas diferencialmente expressas em genótipos de amendoim submetidos a déficit hídrico, caracterizando proteínas de dez grupos funcionais distintos, sendo as mais abundantes as relacionadas com a fotossíntese (25%), lectinas (15%), transdutores de sinal (13%) e estresse hídrico (10%). Curiosamente nos genótipos de maior tolerância verificou-se elevados níveis de proteínas LOX, lipoxygenase e 1L-mioinositol-1-fosfato sintase, um precursor para a síntese do ácido jasmônico, molécula sinalizadora que ativa mecanismos de defesa em plantas e pode estar relacionada com a percepção de sinais

durante o estresse hídrico. Os mesmos autores identificaram aumento da produção de Acetil-CoA carboxilase, uma enzima chave da biossíntese lipídica, que pode estar associada com o aumento de cera epicuticular no genótipo tolerante, sugerindo a identificação de rotas metabólicas que culminam em alterações morfológicas relacionadas com a conservação da água nos genótipos tolerantes.

Ao avaliar as modificações fisiológicas e proteômicas de amendoim, Aminifar et al. (2013) ressaltaram que a tolerância à seca é um processo complexo, e que, portanto, não deve estar sob o controle de um único gene. Por isso é aconselhável combinar o melhoramento convencional com estratégias de engenharia genética, a partir da introdução de genes que aumentem a tolerância destes genótipos a tais condições, uma vez que diversas variedades com alto rendimento e níveis potenciais e moderados de resistência têm sido criadas e liberadas para o cultivo em todo mundo.

Outra estratégia estudada a partir da proteômica tem sido investigar os fatores fisiológicos, ambientais e moleculares que influenciam no desenvolvimento e enchimento das vagens do amendoim. Neste contexto, Zhu et al. (2013) realizaram análises proteômicas comparativa do desenvolvimento de vagens de uma cultivar de amendoim (Yueyou 7), por meio de 2-DE e MALDI-TOF, e identificaram 31 proteínas diferenciais envolvidas em doze processos biológicos importantes: fotossíntese, resposta ao estresse oxidativo, síntese de lignina, biossíntese de ácidos graxos, glicólise, proteína processo catabólico, entre outras.

Portanto, o estudo das interações genéticas, bioquímicas, fisiológicas e agronômicas tem contribuído, para ampliar o conhecimento das rotas metabólicas das plantas e na identificação de genótipos adaptados às condições adversas de ambiente, o que é um fator importante para manter a produtividade estável das culturas, Diante disso, objetivou-se com este trabalho conhecer as respostas fisiológicas, bioquímicas, agronômica e molecular em amendoim submetido a déficit hídrico, visando à identificação de genótipos tolerantes a seca.

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CAPÍTULO II

Selection of drought tolerant peanut genotypes based on multivariate methods

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Selection of drought tolerant peanut genotypes based on multivariate methods

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Abstract

Peanut divergent genotypes were investigated under water stress in order to select five top lines and three cultivars tolerant to drought based on multivariate methods. Plants were submitted to 6d of water suppression and evaluated through physiological, biochemical and agronomical traits. Further, all traits were used to genetic analysis using canonical variables (VCA) and UPGMA multivariate methods. Four groups were formed by graphical dispersion. They were differentiated by earliness and botanical type. In UPGMA analysis, however, only two groups were formed, which genotypes were separated just due to earliness. The CNPA 166 AM, an upright and earliness top line from Spanish type showed better adjustment in physiological, biochemical and agronomical traits. This is a promising material to breeding works focused on semiarid environment. Among runner lines, BR1xLV(B) showed moderate behavior based on performance under water stress conditions. An additional investment in this material could be an interesting strategy for further release of runner cultivar to semiarid region.

Keywords: *Arachis hypogaea*. Antioxidative enzymes. Cell adjustment. Physiological adaptation.

Abbreviations: CO_2 – carbon dioxide; $O_2^{\cdot}$ - superoxide radical; OH - hidroxyl radical; $^1O_2$ - singlet oxygen; H_2O_2 - hidrogen peroxide; ROS - reative oxygen species; SOD - superoxide dismutase; CAT – catalase; APX - ascorbate peroxidase; NBT - nitro-blue tetrazolium; POX – peroxidase; ANOVA - analysis of variance; UPGMA - unweighted pair group method with arithmetic mean; CVA- canonical variables analysis;

mahalanobis distance - D^2, dae- days after emergence, RAH- Relative air humidity, PAR- photosynthetically active radiation

Introduction

Drought is a phenomenon occurring in several regions around the world and is usually associated with other stressing factors to plants, such as high temperatures and salinity, leading to direct effects in growth and development machineries. Plants respond differently to environmental stress depending on tolerance level and life cycle. The identification of genotypes with adjustment to these conditions represents a broad contribution to breeding program focused in arid or semiarid environments.

The fast osmotic adjustment of plants prone to water stress is an essential response to guide the tolerance machinery, because it leads to several secondary processes that could contribute to eliminate or minimize the cell damages (Azevedo Neto et al. 2009; Bolat et al. 2014; Nawaz et al. 2015). The reduction on photosynthesis rate, due to adjustment in stomata conductance, leads to changes in internal CO_2 concentration and further, in efficient use of water, with subsequent effects on crop yield. When associated to high temperatures, the physiological impact in plants submitted to water stress is higher due to increasing on photorespiration rate and spreading of excessive ROS in cells, produced as a result of super excitation of chlorophyll, generating $^1O_2$, $O_2^{\cdot}$, OH and H_2O_2 (Sharma et al. 2012; Ashraf and Harris, 2013; Barbosa et al. 2014). These molecules are quite harmful to cells due its unpaired electrons. Besides, they may sharing other reactions in chloroplasts, mitochondria and peroxisomes leading to oxidative damages, such as lipid peroxidation, protein oxidation, enzyme inhibition, programmed cell death, and others (Sharma et al. 2012).

As defense to ROS, plants produce antioxidant enzymes specialized in cell protection, in different compartments. The most known system involves the event cascades started by SOD, that catalyzes the $O_2^{\cdot}$ dismutation and releasing H_2O_2 and O_2. Further, CAT and APX act in order to eliminate H_2O_2. APX acts in presence of a reducing agent (ascorbate) in compartments CAT-free (Barbosa et al. 2014). Several reports are available in literature evidencing the applicability of these enzymatic systems in order to aid on identification of plants tolerant to drought (Bolat et al. 2014; Nawaz et al. 2015; Pereira et al. 2015).

Peanut (*Arachis hypogaea* L.) is an important oilseed crop, known by its broad adaptation to different environments, including semiarid climates (Nautiyal et al. 2012; Kalariya et al. 2013; Pereira et al. 2016). The species comprises two subspecies (*fastigiata* and *hypogaea*) whose genotypes differ in several morphological, physiological and agronomical attributes. Genotypes from subsp. *fastigiata* are often short cycles and more adapted to dry environments, although they are also vulnerable to drought depending on intensity and reproductive phase. The combination of *fastigiata* and *hypogaea* genotypes in crossing-breeding program has provided promising progenies, with broad variability for further selection procedures in dry environments (Vasconcelos et al. 2015; Ramos et al. 2015; Pereira et al. 2015). In this work, we submitted peanut divergent genotypes to water stress in order to identify tolerant materials based on multivariate methods, from physiological, biochemical and agronomical traits.

Material and Methods

Genetic resources and experimental procedures

Eight genotypes (five top lines and three cultivars) from Peanut Breeding Program coordinated at Embrapa were kindly provided for this study (Table 1). Top lines are promising genotypes, selected based on high yield and seed quality obtained in field assays.

Table 1 Descriptive characteristics of genotypes used

Genotype	Origin	Genealogy	HC	TB	TS	S/V
1- Senegal-55437	Africa	Cultivar	Erect	Spanish	Small	2
2- LViPE-06	Brazil	Top line	Runner	Virginia	Extra large	1-2
3- BR 1	Brazil	Cultivar	Erect	Valencia	Medium	3-4
4- BR1 x LViPE-06(B)	Brazil	Top line	Runner	Virginia	Large	1-2
5- BR1 x LViPE-06(V)	Brazil	Top line	Runner	Virginia	Large	1-2
6- BRS 151 L7	Brazil	Cultivar	Erect	Valencia	Large	3-4
7- LGoPE-06	Brazil	Top line	Runner	Virginia	Extra large	1-2
8-CNPA166 AM	Africa	Top line	Erect	Spanish	Medium	1-2

HC: Growth Habit; TB: botanical type; TS: Seed size; S / V: number of seeds per pod.

Seeds were grown in greenhouse and submitted to short water stress. The experimental procedure was carried out according to Azevedo Neto et al. (2009). Four seeds of each genotypes were sown in pots (10 L) containing sandy-loam texture soil previously limed and fertilized (NPK, 20:60:30, ammonium sulfate, single superphosphate and potassium chloride). At 21 dae, seedling were thinned to 2 per pot. The watering (100% field capacity) was daily until seedlings aged 20 d. Then, treatments were established as: control (100% field capacity) and stress (total drawal of water during 6 d). Field capacity was determined by gravimetric method after 72 h of draining. The pots of both treatments were weighed daily and, in the control treatment, the water lost by transpiration was replaced (Pereira et al. 2016). In order to prevent the losses by evaporation, soil surface of each pot was covered with polyethylene discs. A completely randomized design was adopted, with 2 treatments and 10 replications. After water stress, plants were normally watered before harvest. Upright and runner genotypes were harvested at 85 and 120 dae, respectively. The traits collected in each genotype/treatment were: weight of 100 pods (g), weight of 100 seeds (g), plant height (cm), blooming (dae), full pod maturation (dae) and number of pods per plant. Data were further used to statistical analyses.

Gas exchanges determination

Gas exchanges were estimated by an infra-red gas analyzer (IRGA) (Li-Cor 6400, Lincoln, Nebraska, EUA), after 6 d of water stress, in interval of 8:00-10:00 h, from leaves located at upper canopy. CO_2 concentration was adjusted to 380 µmol mol^{-1}. RAH varied from 53% to 66% during assay, and PAR was set on 1.200 mol $m^{-2}s^{-1}$.

The registrated physiological parameters were net photosynthesis (A, µmol $m^{-2}s^{-1}$), stomatal conductance (gs, mol $m^{-2}s^{-1}$), internal CO_2 concentration (Ci, µmol mol^{-1}) and transpiration (T, mmol $m^{-2}s^{-1}$). Additionally, were also estimated the water usage efficiency (WUE) from A/T ratio and the instantaneous carboxilation efficiency (EiC)

from A/Ci ratio. All measurements were performed in triplicates and the average values were used used in statistical analyzes.

Antioxidant enzymes activity

At the end of water stress period, leaf samples were harvested under refrigerated conditions to antioxidant enzymes analysis: SOD, APX and CAT. Samples were processed for total protein extraction (2.5%), using dibasic potassium phosphate buffer (0.1 M), pH 7.5, containing 100 mM EDTA, 100 mM L-ascorbic acid and 4% PVP (w/v). All the samples were quantified by Bradford method (1976).

SOD activity was determined in accordance to the method described by Bulbovas et al. (2005), with exposure to fluorescent light (75 W) for 15 min and absorbance read at 560 nm. One unit (U) of SOD is needed to inhibit 50% of NBT (nitro-blue tetrazolium) substrate reduction. APX was measured from H_2O_2 decomposition at 290 nm, adopting the coefficient of molar extinction of 2.8 mM^{-1} cm^{-1} ascorbate (Nakano and Asada, 1981). CAT was determined according to Beers Junior and Sizer (1952) with few modifications: 80 mM H_2O_2 and 100 µL protein extract. Activity was calculated from the absorbance difference at every 15 s intervals in 1 min. The molar extinction coefficient of 39.4 mM^{-1} cm^{-1} was used and the absorbance read at 240 nm. The absorbance change of 0.01 U min^{-1} was considered as equal to 1 CAT unity.

Statistical analysis

Physiological, biochemical and agronomical data were used in normality test (Lilliefors) and then for variance analysis by F test and average comparison by Tukey test ($p \leq 0.05$). The selection of water deficit tolerant lineages was based on similarity tests, adopting methods of multivariate grouping. Genetic distance between every genotypes pairs was estimated through Mahalanobis generalized distance (D^2) and canonical variable analysis method. From D^2, the UPGMA grouping method was applied. To assess the accuracy of clustering, the cophenetic correlation coefficient (CCC) was calculated, obtained from 1,000 simulations and analyzed by t test. All statistical analyses were performed with program GENES 2015.5.0 (Cruz, 2013).

Results and Discussion

Five peanut top lines were submitted to 6 d of water suppression, at previous blooming, and further evaluated to water stress tolerance based on physiological, biochemical and agronomical features. Three cultivars were used as control: BR 1, an upright and short cycle, recommended to semiarid environment (Vasconcelos et al. 2015; Gomes et al. 2007), Senegal-55437, an earliness and tolerant to drought (Azevedo Neto et al. 2009) and the genotypes selection was conducted by multivariate methods.

Variance analysis revealed significant statistical difference for all genotypes (G) and treatments (RH) in studied physiological, biochemical and agronomical variables. The effect of G X RH interaction was verified for most variables, indicating that the genotypes responded differently to the treatments. Exceptions were observed in initial flowering and complete pod maturation phases. In following, it is presented a genotypes behavior analysis.

Gas exchange profile in drought stressed plants

The effect of water suppression over plants was first observed in short (Runner type) lineages, with notable reduction in leaves color and turgidity during water suppression period (Fig 1). The recovery of these plants, in vegetative growth terms, was also slow even after re-watering.

Fig. 1 Detail of Senegal 55 437 (A) and LViPE-06 (B) genotypes, respectively tolerant (left) and sensitive (right) to drought, after 6 days of water suppression in greenhouse

In Fig 2 it is shown the result of gas exchanges in evaluated lineages, in normal and suppressed watering condition for 6 days. As expected, the resistant cv. Senegal-55437 had 90% lowering in gs (Fig 2A), indicating that the plant was able to sense the cell damage derived from lack of water and rapidly responded by closing stomata, consequently reducing the water loss by T (Fig 2B). In the remaining materials such behavior was also observed, although gs reduction has been close to 64%. The exception was the Runner type late cv. LViPE-06 which also had the ability of quick stomatal closure (80%) and transpiration decrease; this could be initially considered a strategy to escape from water deficit stress. According to Pereira et al. (2016), stomatal closure in response to adverse water conditions have been described as one of the first physiological changes in peanut plants. It represents a direct advantage to prevent dehydration due to transpiration reduction (Taiz and Zieger, 2004),

In spite of it, and based on Ci data (Fig 2C), elevated values were observed in most of short (Runner type) lineages, including LViPE-06. Ci represents the internal cell CO_2 concentration, needed for physiological processes related to photosynthate formation. Thus, considering the obtained results, it is concluded that top short lines were more sensitive to water deficit, since the high CO_2 concentrations may have resulted from photorespiration increase justified by significant photosynthesis rate (A) decrease (Fig 2D). These results corroborate those obtained by Erismann et al. (2006), who evaluated the gas exchanges in short peanut at vegetative and reproductive phases, and verified that A tend to increase along with Ci increase, if there is no photorespiration elevation that could release more CO_2 and so to reduce the net CO_2 assimilation rate.

The water usage efficiency (WUE) refers to the plant ability of using the available water efficiently. According to Fig 2E, it was demonstrated that the short (Runner type) lines, despite being highly productive, present low water usage efficiency, which can be explained by their longer cycle and land area cover. The CNPA 166 AM line presented the best adjustment to imposed conditions, considering its

physiological profile for gas exchanges where gs and T were decreased (Fig 2A and 2B), maintaining the CO_2 content (Fig 2C) balance to photosynthate production enough to keep A (Fig 2D). Besides, the plant kept high EiC (Fig 2F), indicating that CO_2 was fixed for biomass production and maintenance of subsequent physiological processes. Such adjustment allowed it to present a similar to the BR 1, which is reported as one of the cultivars most adapted to semi-arid environment (Gomes et al. 2007; Santos et al. 2010).

Antioxidant activity of genotypes due to water stress

Water suppression on peanut plants resulted in biochemical stress revealed by antioxidant complex enzymes. SOD, CAT and APX activities were used to estimate the reduction of H_2O_2 derived from oxidative damage (Fig. 3). Since SOD constitutes the first defense step, acting on superoxide anion (highly toxic to cell) dismutation, the increase of the enzyme in stressed plants is a natural occurrence as a way to transform the $O2^{\bullet-}$ excess into H_2O_2 and O_2, for further neutralization of these ROS by CAT and APX, who degrade H_2O_2 into H_2O and O_2. As shown in Fig. 3A, all the genotypes presented an elevated SOD input, with equal or higher levels than control plants. However in concentration terms, the short LViPE-06 revealed low levels in both situations, what may change defense process since SOD stock contributes to keep membrane permeability and also to overcome the formation of $^{\bullet}OH$ radical, very reactive (Sharma et al. 2012).

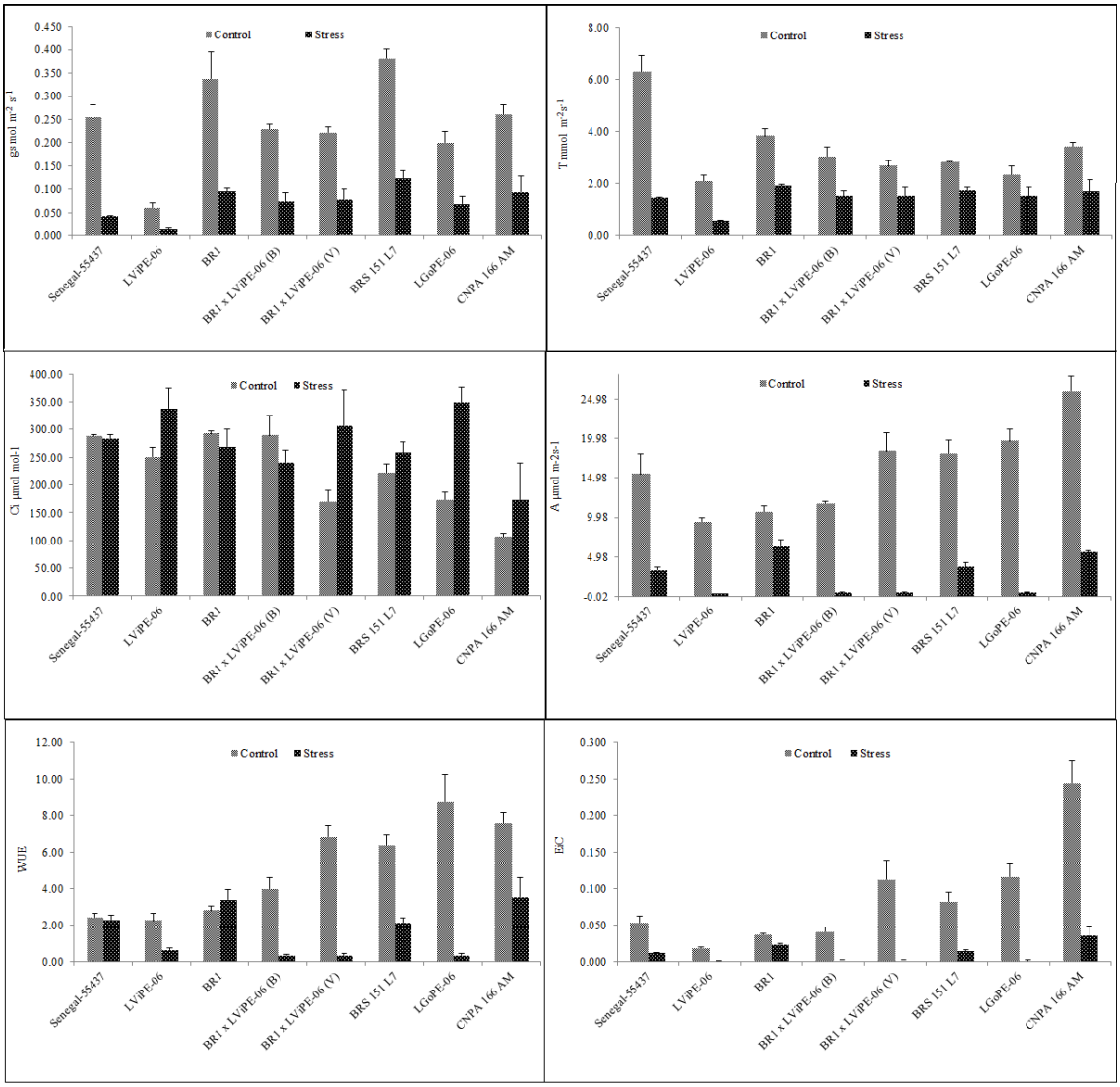

Fig. 2 Physiological variables relative to peanut gas exchanges. Values were collected with IRGA after 6 days of total water suppression

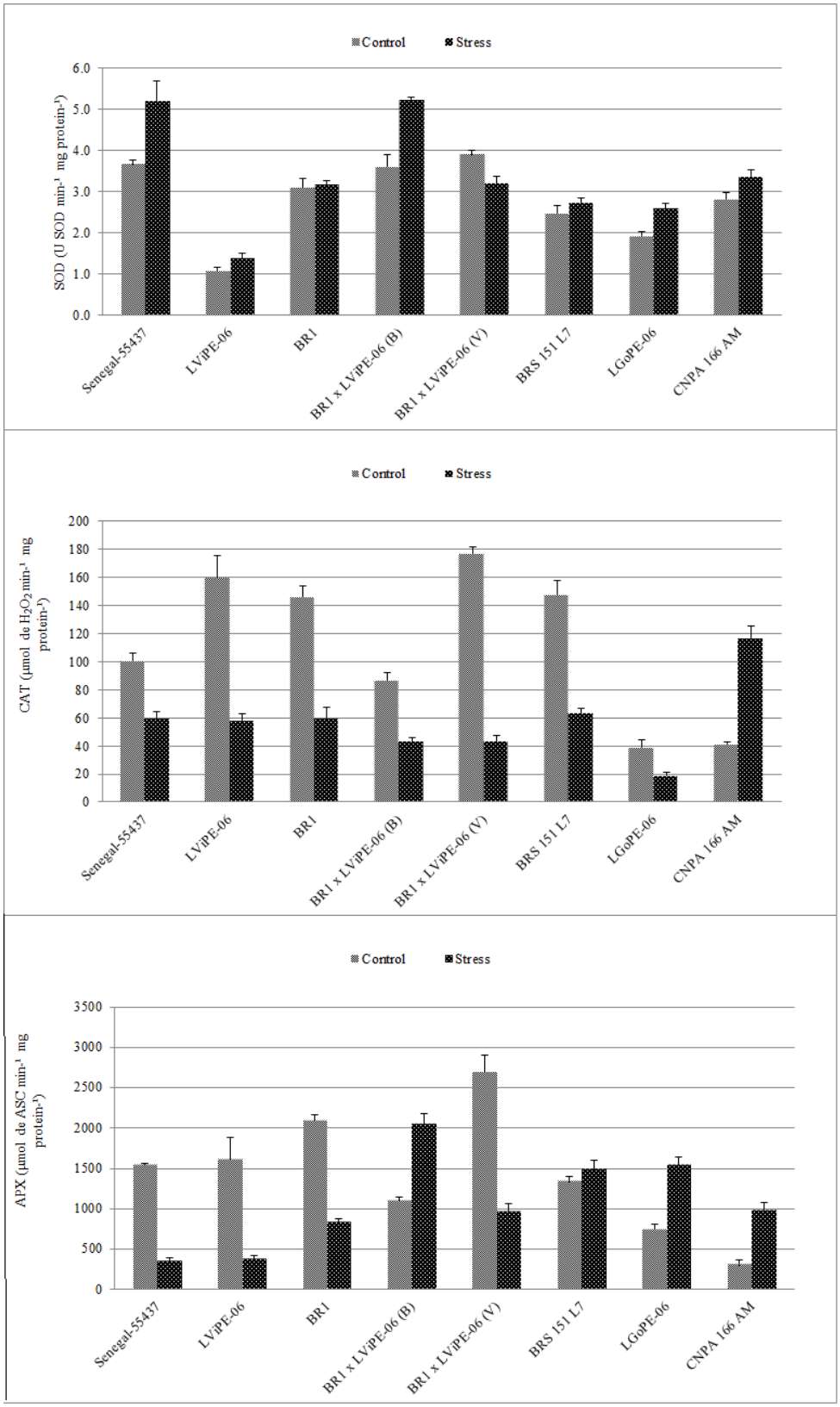

Fig. 3 Activity of SOD, CAT and APX enzymes in peanut genotypes submitted to 6 days of water suppression

As consequence of SOD action, CAT neutralizes H_2O_2 produced by oxidative process. It is noted in Fig 3B that all genotypes presented a reduction in this enzyme activity, except CNPA 166 AM which had a more increased action, allowing to infer it

is a differentiated detoxification process, considering the genealogy of this top line that is derived from a pyramidal crossing involving eight progenitors of three distinct botanical types (Santos et al. 2013).

For the residual peroxides left in cell, APX acts neutralizing remaining molecules as a way to minimize or eliminate cell losses caused by water stress. Noteworthy, the cultivars Senegal-55 437, LViPE-06, BR 1 and CNPA 166 AM revealed lower enzyme concentrations (Fig 3C). However, considering the integrated machinery of events cascade that involves these three enzymes, it is concluded that Senegal-55 437, BR 1 and CNPA 166 AM are the most adapted in biochemical terms. In accordance to Nawaz et al. (2015), the osmotic adjustment develops slowly, while the antioxidant enzymes activity may occur immediately as ROS are produced in excess in plant cells, thus promoting the adaptation to adverse environmental conditions. The roles of CAT and APX are essential due to the ability to identify ROS accumulation in each cell compartment. According to Akcay et al. (2010), APX is responsible for fine modulation of antioxidant response. Once CAT does not require reducing factors to accomplish its functions, it may be insensitive to the cell redox status during stress and the combination of both may provide a better protection to cells when stress sets.

Agronomical characteristics

Significant statistical difference was verified among all genotypes and treatments for all variables. The effect of G x RH interaction was also verified, except for the initial flowering and pode maturation phases, indicating that these variables were not influenced by the water deficit imposed to plants.

The erect genotypes were less affected by water stress, with losses in yield characters below 22% (Table 2). The top line CNPA 166 AM behaved as more tolerant since it revealed minimal yield loss, quite similar to its genitor Senegal 55-437.

Among the short ones, the top lines BR1 x LViPE-06(B) and BR1 x LViPE-06(V) revealed intermediate performance compared to their genitors and, so, presented lower yield loss percentages than the late short genotypes LViPE-06 and LGoPE-06. This ability, possibly benefiting from maternal inheritance of BR 1, places them as promising genotypes to be furthered in breeding towards low water availability environments. Such interference is based on the study of Silva et al. (2016), who estimated the genetic divergence in peanut short (Runner type) genotypes and verified that the lines generated from the cross between BR 1 and LViPE-06 fit inside a group of short genotypes with precocious and intermediate profiles. This information is relevant because it opens perspective to establish these two lines in semi-arid environments, considering that most of cultivated commercial short cultivars in Brazil are sensitive to such climate and have very long cycles (120-140 days) (Santos et al. 2012). On the other hand, it stands out that one of the great benefits of short cultivars in Northeastern is the productivity, although they demand more water in management since their canopy conformation indicates mechanical harvest.

Table 2 Average agronomic analysis: weight of 100 pods (w100p), weight of 100 seeds (w100s), height and number of pods per plant (pod/pl)

	w100p			w100s		
Genotype	**C**	**E**	**(%)**	**C**	**E**	**(%)**
Senegal-55437	77.40Ad	69.30Bg	-10	40.45Ae	35.6Bg	-12
LViPE-06	172.44Aa	82.77Bb	-52	73.75Ab	35.40Bb	-52
BR1	101.52Ac	84.58Bcd	-17	45.85Ad	38.90Bd	-15
BR1 x LV(B)	105.66Ac	65.66Bc	-38	57.05Ac	44.45Bc	-22
BR1x LV(V)	110.24Ac	70.54Bef	-36	57.62Ac	45.62Bde	-21
BRS 151 L7	122.70Ab	96.9Bde	-21	55.92Ac	42.03Be	-20
LGoPE-06	173.17Aa	91.78Ba	-47	78.32Aa	41.51Ba	-47
CNPA 166 AM	87.42Ad	76.92Bf	-12	46.40Ad	41.76Bf	-10
	Height			**pod/pl**		
Genotype	**C**	**E**	**(%)**	**C**	**E**	**(%)**
Senegal- 55437	30.00Ab	24.85Bb	-17	19.50Ad	16.7Bf	-15
LViPE-06	9.45Ad	5.47Bd	-42	45.75Aa	21.96Bb	-52
BR1	42.20Aa	32.30Ba	-23	25.00Ac	19.50Bd	-22
BR1 x LV(B)	21.02Ac	16.05Bc	-24	32.50Ab	22.8.0Bc	-30
BR1x LV(V)	21.50Ac	16.95Bc	-21	36.00Ab	25.44Bd	-31
BRS 151 L7	44.60Aa	34.85Ba	-22	25.75Ac	19.95Be	-21
LGoPE-06	9.45Ad	6.47Ab	-32	49.50Aa	26.23Ba	-47
CNPA 166 AM	34.90Ab	24.87Bd	-29	19.25Ad	12.25Bef	-18

Means followed by the same letters, uppercase and lowercase between water treatments between genotypes do not differ statistically by Tukey test ($p < 0.05$); C: control; E: Stress; (%) Percentage Difference

Regarding the height, all plants had growth reduction ranging from 17% to 42%. Such lowering does not configure, necessarily, an undesirable feature: since the gynophore of erect plants is located on the main stem, the small size facilitates the penetration of the gynophores into the soil and, later, the development of pods. The damage, however, derives from how much this size reduction might also be associated with yield loss, which has been observed in the performance of LViPE-06 and LGoPE-06 who had their height reduced in 42% and 32%, respectively, with reflex on pods production characters.

Clustering analysis by multivariate methods

Based on physiological, biochemical and agronomical data obtained from stress treatment, it was performed a multivariate analysis through CVA and UPGMA methods. Distance matrix between accesses was generated from D^2 values. For CVA, it was verified that the two first canonical variables explained approximately 87.26% of total accumulated variance (CV1 = 60.06%; CV2 = 27.20%) (Fig 4), pointing out that most of the variability is summarized in the two first components and the genotypes classification may be represented in a bidimensional graph.

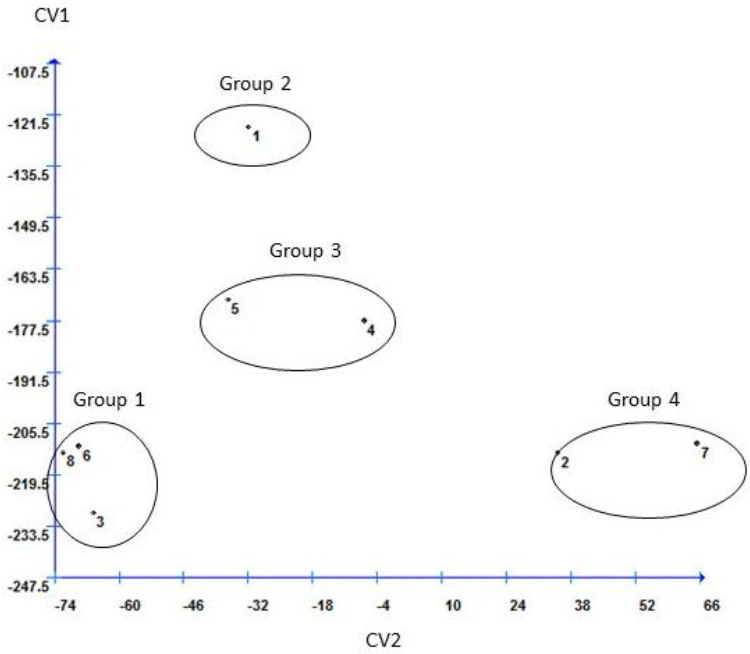

Fig. 4 Graphical dispersion of scores in relation to first two canonical variables, based on seventeen traits obtained in the 8 peanut genotypes. 1- Senegal-55437, 2-LViPE-06, 3- BR1, 4- BR1xLViPE-06 (B), 5- BR1xLViPE-06 (V), 6- BRS151 L7, 7- LGoPE-06 and 8- CNPA 166 AM

Four clusters were formed with the following compositions (Fig 4): G1 represented by Valencia-type genotypes, erect and precocious - BR 1 (3), BRS 151 L7 (6) and CNPA 166 AM (8); G2 composed only by the drought-resistant cultivar Senegal-55437 (1), Spanish-type, erect and precocious; G3 composed by top lines BR1xLViPE-06(B) (4) and BR1xLViPE-06(V) (5), both short-type with intermediate cycle, dense canopy and descent from BR 1 x LViPE-06; and G4 represented by late short ones LViPE-06 (2) and LGoPE-06 (7), both Virginia-type. In the classification after UPGMA analysis, only two clusters were formed considering a dissimilarity index of 77%, proposed by Mojena (1977) (Fig 5).

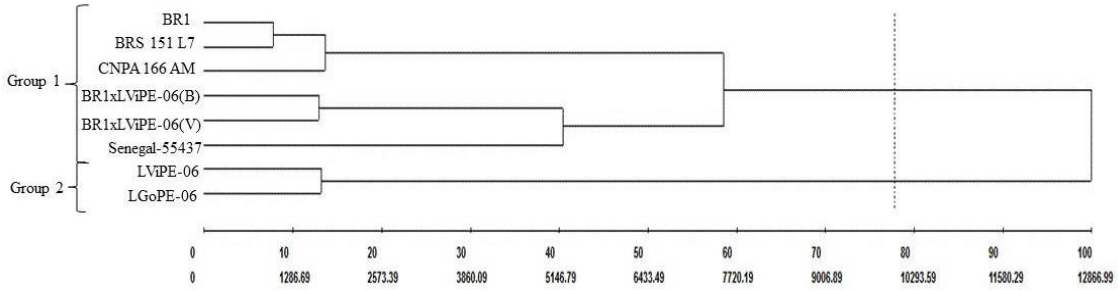

Fig. 5 Genetic divergence in peanut subjected to 6 days of water deficit by UPGMA method

The cophenetic coefficient was 0.77%. In this way, the UPGMA-derived dendrogram configuration was more rigid, separating the genotypes in just two classes:

precocious-intermediate and late. The CVA-derived classification was more contributive to breeders because it showed three situations where the genotypes can be selected in genetic improvement works, depending on specific program demands.

Conclusions

Based on physiological, biochemical and agronomical descriptors adopted to classify peanut genotypes with tolerance to water deficit by multivariate methods, it was found that the top line CNPA 166 AM was identified as the most tolerant genotype to the submitted water stress.

Acknowledgements

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CAPÍTULO III

Peptides accumulated in peanut submitted to water stress

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Peptides accumulated in peanut submitted to water stress

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Abstract

Drought is an important environmental stress that is faced up by several crops, at worldwide. The effect lead to changes in plant growth and limit its production. Peanut is a short cycle crop that shows different responses when grown in environments prone to drought. To semiarid climates, the adoption of drought tolerance cultivars is the best option to the farmers. Several methodologies are available in order to identify drought

tolerant genotypes. In this work, two contrasting peanut genotypes were submitted to a short water stress and evaluated by proteome tools in order to identify proteins associated with drought tolerance. A total of 52 differentially expressed proteins were found, among them 32 were identified in water-stressed treatment, and six were directly involved in routes of drought tolerance. Proteins were involved in redox homeostasis, photosynthesis, beta-oxidation and osmoprotection. Six protein were found, all of them involved in several biotic and abiotic processes, including drought tolerance: Kinesin-like, Enoyl-CoA hydratase, Photosystem I subunit, AP2/ERF transcription factor, DNA helicase and Defensin-like protein, This study provides new insights into understanding of the molecular mechanism of tolerance to drought, and could help to assist in selection procedures in peanut breeding focused on development of varieties to semiarid environment.

Keywords: *Arachis hypogaea*. Abiotic stress. Mass spectrometry. Functional classification. Tolerance.

Abbreviations

ABA - Abscisic acid

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CAC - Clathrin adapter complex

CAPES - Coordination for the Improvement of Higher Education Personnel

CBB - Coomassie brilliant blue

CETENE - Center of Strategical Technologies of Northeast

CHAPS - 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

dae - days after emergence

DREB - Dehydration-responsive element binding

DTT - 1,4-dithiothreitol

DTT - Dithiothreitol

EB - equilibration buffer

ERF8 - Ethylene Responsive Factors

GLU -TR - Glutamyl-tRNA reductase

H_2O_2 - Hydrogen peroxide

IAA - Iodoacetamide

LCFAs - Long-chain fatty acids

LEA - late embryogenesis abundant proteins

MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry

MeJA - Methyl jasmonate (MeJA)

PLP synthase - Pyridoxal 5'-phosphate synthase

PMF - peptide mass fingerprinting

PPIase - Peptidyl-prolyl cis-trans-isomerase

ROS - reactive oxygen species

SA - Ethylene, salicylic acid

SDS - Sodium dodecylsulfate-polyacrylamide

TFA - Trifluoroacetic acid

TXS - Taraxerol synthase

UDP-galactose - β-1,3-galactosyltransferase

1DGE - one-dimensional gel electrophoresis

2DGE - two-dimensional gel electrophoresis

1. Introduction

Climate changes have caused drastic damages to agricultural production, reflecting directly in economic losses to several commercial crops. Water scarcity is one of the most serious consequences of environmental changes because affects the growth and development of crops, especially in reproductive stage. Plants have different levels of tolerance to water deficit, which could be estimated by physiological or biochemistry assays. Additionally, molecular tools have been used to assist this information for further use in crops breeding, especially in programs focusing on semiarid environments.

Drought is one of the most important abiotic stresses that causes expressive lost of yield in several crops. The inheritance to drought resistance is multigenic, incompletely penetrant and quantitative (Govind et al., 2009). Such factors make difficult the selection procedures often adopted in conventional crop breeding.

The molecular base of drought tolerance is quite complex since it involves signaling cascades, leading to activation or suppression of several genes, related to different pathway associated to dissection perception. The whole machinery works coordinately in order to address signals to retain water and avoid dehydration. Although

some mechanism are common to all cells, there are differences in strategy adopted by plants to cope with desiccation, at cell level.

Genomic and proteomic studies have depicted the nature of genes and their products, providing expressive resources for crop breeding. Genes responsive to water stress from tolerant plants provide better protection do cell structures due to existence of genes that code to specific proteins associated to stress adaptation (Govind et al., 2009). Taking in account the often-weak correlation between mRNA and protein abundance, proteomics techniques provide reliable information to functional analysis of translated regions of the genome (Hossain and Komatsu, 2014).

Peanut (*Arachis hypogaea* L.) is an important oilseed crop, known by wide adaptation to several environments, especially in fastigiata subsp. accessions (Valencia and Spanish botanic types) (Pereira et al., 2016). Despite to this ability, some genotypes are quite sensitive to drought stress in flowering and pod filling, affecting the yield, depending on the intensity.

Several molecular tools have been used to screen drought tolerance in peanut genotypes, generating genomic and proteomic data useful to understanding of the tolerance mechanism for further use in selection procedures of crop improvement. An expressive study related to drought stress was carried out by (Jain et al., 2001) that used subtractive library in tolerant and sensitive peanut and found over than 1,200 and 950 differential display products in irrigated and water stressed plants, respectively. In a transcriptome-proteomic study, (Kottapalli et al., 2009) evaluated a peanut mini-core collection under water-deficit stress and found forty-nine non-redundant proteins, implicating a variety of stress response mechanisms. According to authors, lipoxygenase and 1l-myo-inositol-1-phosphate synthase, which aid in inter- and intracellular stress signaling, were more abundant in tolerant genotypes under water-deficit stress. Also, they reported a marked decrease in amount of several photosynthetic proteins in tolerant genotype. These results are quite important to further physiological and biochemical assays, focusing on selection of drought tolerant genotypes.

In studies involving the response of plants tolerant to water stress, it is convenient to use a combination of biochemical and physiological measurements, associated to molecular tools, which responses represent the initial step of several changes that takes place in cell machinery.

Literature reports a sum of articles focused on response of peanut submitted to water stress in reproductive phase (Chakraborty et al., 2015; Pereira et al., 2016).

Although these results have contributed to a better understanding of the consequences of stress in the stages of flowering and filling of the pods, there is the possibility of some defense pathways are redundant, since peanut plant has a determined cycle. Thus, prior knowledge of the molecular events that the cell triggers at onset of stress may contribute as strategy to plant defense and in further selection procedures adopted in crop breeding.

In this study we used proteomic tolls in order to identify proteins associated to drought tolerance, based on two contrasting peanut genotypes submitted to a short water stress, in greenhouse. The basis of this study was focused on translation levels, which were analyzed by 1DE and 2DE.

2. Materials and Methods

2.1 Genetic resources and experimental procedure

Two genotypes previously phenotyped to drought tolerance were used in this work: Senegal-55437 (*A. hypogaea* subsp. fastigiata, var. vulgaris) and LViPE-06 (*A. hypogaea* subsp. hypogaea, var. hypogaea). Seeds were sown in pots (10 L), containing commercial substrate (Plantmax), in greenhouse and watered daily, maintaining field capacity. At 21 dae, only two plants were kept in pots and treatments were differentiated as control: (C), plants watered daily, and stress (S): total water suppression for 6 d, when stomatal conductance was reduced in 80%. Treatments were completely randomized with 4 repetitions. After period of water suppression, young leaves were collected to molecular assays.

i.*2.2 Protein extraction and SDS-PAGE*

Samples from young leaves of each genotype/ treatment were used for the proteomic analyses. The total protein extraction procedure was performed as described by (Pacheco et al., 2013), using phenolic method. The leaf tissue was homogenized in liquid nitrogen and extracted with 20% trichloroacetic acid in acetone.

For the 1DGE analysis, the total proteins were extracted from the dried precipitates with a lysis solution containing urea, CHAPS and DTT. The protein concentration in extracts was measured using the Bradford method with bovine serum

albumin as the calibration standard (Bradford, 1976). SDS gel electrophoresis was used to separate proteins, further stained with CBB G-250. Differential spots in both treatments were isolated and analyzed by MALDI-TOF – MS, after trypsin cleavage.

2-DGE was performed using immobilized pH gradient (IPG) strips (pH 4–7, nonlinear, 11-cm). The IPG strips were rehydrated overnight in 250 μL rehydration buffer [7M urea; 2M Thioureia ; 4% (w/v) CHAPS; 20 mM DTT; 2% (w/v) IPG buffer, pH 4–7; bromophenol blue]. Each strip was loaded with 150 μg protein. The proteins were submitted to isoelectric focusing at 20°C in a Multiphor II horizontal electrophoresis unit (GE Healthcare, Piscataway, NJ, USA). After isoelectric focusing, the IPG strips were equilibrated in EB [7 M urea, 2M Thioureia, 2% (w/v) CHAPS, 2% (v/v) IPG buffer, 0.002% (w/v) bromophenol blue pH 8.8, containing 1% (w/v) DTT] during 20 min, and then alkylated in EB containing 2.5% (w/v) IAA, for 20 min.

The strips were transferred to 12.5% SDS-PAGE gels with no stacking gel. The proteins were then electrophoretically separated at 20°C at 250 V 45 mA. The 2-DE gels were stained in CBB G-250 and further scanned using a GS-800 calibrated densitometer. All assays were performed in three independent replicates.

The analyses were done by using ImageMaster software (GE Life Sciences). Differential spots in both treatments were isolated and analyzed by MALDI-TOF MS, after trypsin cleavage. The follows parameters were adopted: partial threshold, 4; saliency, 2.0; minimum area, 50. In order to verify the auto detected results, all spots were manually quantified by ratio: volume of each detected spot/total volume of all spots on the gels (Pacheco et al., 2013).

2.3 Trypsin digestion and MS analysis

Gel fragments containing differentially accumulated bands (protein mixtures) were manually excised, detained in 50% methanol and 2.5% acetic acid solution for 3 h at room temperature. Then they were submitted to dehydration in 100% acetonitrile solution. In sequence, protein samples were reduced with DTT (10 mM) during 30 min and then alkylated in IAA (50 mM) for 30 min. Furher, each fragment was washed in ammonium bicarbonate (100 mM) and dehydrated (3x) with acetonitrile (100%). Trypsin (20 ng μL^{-1}) was then added and samples were incubated at 37ºC overnight. After removal of trypsin buffer excess, TFA 5% in acetonitrile 50% was added and

samples were incubated at 4ºC/1 h. The samples were centrifuged and the supernatant collected for further MS analysis.

Analyses were performed for fragmented protein samples in AutoFlex III ToF/ToF automatic analyzer (Bruker Daltonics, Bremen, Germany), through MALDI-TOF MS. All these analyses were carried out at CETENE, Recife-PE, Brazil. Protein mixtures-derived peptides were eluted in matrix solution (1% α-cyano-4-hydrocinammic acid) containing TFA (1%). Mass spectra were obtained according to PMF, according to reflexive method (RP_Proteomics), excluding ions with *m/z* ratio ≤ 700 Da, and analyzed with program Flex Analysis (Bruker).

2.4 Putative identification of proteins

Peptides mass spectra were used for putative identification of corresponding proteins by using the online version of program MASCOT (http://www.matrixscience.com/search_form_select.html), set for Swissprot/Viridiplantae database (http://www.uniprot.org/). A significant match score ($p \leq 0.05$) was adopted to annotated protein sequences. A unique UniProt accession was used in further functional analyses (UniProtKB, http://www.uniprot.org/).

3. Results and Discussion

Peanut plants, sensitive and tolerant to drought, were submitted to 6 d of water suppression and assayed for differential expression based on 1 and 2-DE tools. During growth, visible changes were detected in plants after 6d of drought. Low turgor-leaves were verified and also with reduced height of main axis. Even in a low condition of water stress, LViPE-06 were more sensitive, with visible aspect of dehydration and slowness in growth (Fig. 1). These aspects were also seen in others reports, soon at early growth, in both moderate and severe water stress condition, with deep changes in physiological and agronomical traits (Santos et al., 2010; Pereira et al., 2012; Duarte et al., 2013; Pereira et al., 2016). This information is relevant because the physiological condition of tester provides security to attest the results generated in further proteomic assays.

Fig. 1. Aspect of Senegal-55437 (A) and LViPE-06 (B) plants submitted to 6d- water suppression. C- control; S- stressed.

3.1 Protein identified during 6d- water suppression in sensitive and tolerant peanut genotypes by 1-DE SDS-PAGE

Changes in protein profiles were seen in both tolerant and sensitive genotypes by 1-DE SDS-PAGE. Forty bands were obtained from plants in both treatments, which were analyzed through MS, ranging from 200 to 10 kDa. Among them, 22 bands were differentially identified, although no directly involved with abiotic stress. Putative identification of peptides was achieved according to significant match scores in comparison to Swissprot/Viridiplantae database. Noteworthy, each band is likely to contain several different proteins, but the analysis in MS spectra, normally leads to identification of one or few most abundant peptides, whose high intensity signals impair the detection of other peptides that can be present.

In order to discuss our results, we considered only up- or down-regulated proteins with some involvement to water stress, found in both stressed treatments.

To LViPE-06 we found four proteins, two up-regulated (CAC and GLU-TR) and two down regulated in stressed plants (ATP synthase and TXS). We will briefly describe the involvement of each one in the defense route.

CAC - When cell face up the water stress, several proteins are degraded due to several associated effects, such as elevation of cell temperature, oxidation of enzymes, among others. Clathrin is a chaperone essential during osmotic adjustment due to its involvement in transport of proteins that will be degraded during physiological stress. These protein complexes can contribute to overcome stress in susceptible genotype, since they are involved with the formation of transport vesicles and, under stress, the

cells produce more molecules that will be transported for disposal. CAC is involved in recruiting proteins to clathrin coated vesicles. There is also evidence that adaptors bind to the cytoplasmic domains of selected transmembrane proteins and enable them to become concentrated in the coated vesicle (Hirst and Robinson, 1998). There are evidence that CAC is expressed constitutively, during plant onthogeny. Migocka and Papierniak (2011) evaluated reference genes in cucumber plants submitted to abiotic stress and found that CAC should be the most reliable reference gene for studies on the effect of temporary and permanent heavy metal stress on target gene expression in cucumber roots, shoots and leaves. In *Arabidopsis,* (Czechowski et al., 2005) reported that CAC is stably expressed in *Arabidopsis* roots under abiotic stress.

GLU-TR - is encoded by a gene involved in chlorophyll biosynthesis, in higher plants and respond directly when cells are submitted to environmental stresses, activing or repressing the activity, soon at early growth, or even increasing the activity of its precursors (Stenbaek and Jensen, 2010). In 5-day-old rice seedlings treated with salt (0.5% NaCl) for 48 h, (Wen et al., 2010) found eleven proteins differently regulated by salt, among them a GLU-TR, in a down-regulated condition. Also, Ahsan et al. (2007) found 35 proteins differentially expressed in 5-week-old tomato roots submitted to waterlogging stress. Among them, several were up- regulated and reported as involved in hormone and secondary metabolite synthesis, programmed cell death, and stress and defense mechanisms, such GLU-TR, cysteine protease, 3-beta-hydroxylase, phenylalanine ammonia-lyase, among others.

ATP synthase - This enzyme plays a central role in plants under abiotic stress because attends demands of cell during defense processes, such as energy transduction in chloroplasts and mitochondria, and alleviation of stress. Water suppression leads to substantially decreases CO_2 assimilation by net reduction for ATP in cell machinery (Tezara et al., 1999). Then, the induction of ATP synthesis is necessary to assist in several events associated to abiotic stress tolerance (Zhang et al., 2008). In *Arabidoipsis*, the over-expression of the ATP synthase resulted in greater tolerance to drought (Zhang et al., 2008). Kottapalli et al. (2009) submitted peanut to a short period of water stress and analysed changes in leaf proteins during reproductive stage growth. Forty-nine non-redundant proteins were identified, implicating a variety of stress response mechanisms in peanut. An ATP synthase epsilon chain and an ATP synthase beta subunit were highly induced only in tolerant genotypes suggesting their putative role in water-stress tolerance. According to authors, induction of the protein in tolerant

peanut genotypes may alleviate water-deficit stress by increasing ATP supply to meet increased stress-related energy demand. In sensitive plants, this mechanism is repressed leading to several phenotypical symptoms, depending on level of plant sensibility.

TXS – The cell hole of this enzyme in events associated to abiotic stress is poor reported in commercial crops. In literature, we found that TXS is required to synthesis of taraxerol, an oleanane triterpenoid, present in cuticular waxes and involved in membrane protection processes. Triterpenes are part of the terpenoid family, an extensive group of natural products, which are abundant in the plant kingdom, which compounds possess antifungal and antioxidant properties, among other effects (D'Abrosca et al., 2005; Yuan et al., 2008). The reports about physiological activity of TXS in plant defense systems are also limited, but taking in account the action of terpenoids at cell level, we suggested that the role of TXS in machinery of membrane defense against abiotic and biotic stresses is quite relevant. As all defense process in cell depends on a set of receptors and transmembrane proteins, we suggested that the activity of TXS is repressed in sensitive-stress plants, due to a disordered or even slow system in defense response.

To Senegal-55437, we found several proteins involved with drought tolerance, the most was widely reported in literature, such as phosphatases, peroxidases, cytochrome p450, etc. We stand out in only two up-regulated, UDP-galactose and PPIase, both involved in osmoprotectant events and poorly reported in stressed plants.

UDP-galactose - Raffinose family oligosaccharides (RFOs) have multiple functions in plants and playing a role as compatible solutes in the accumulation during drought stress (Peters et al., 2007). UDP-galactose, has been proposed to be the key enzyme of the RFOs pathway, due to its involvement in galactinol synthesis, an osmoprotector oligossacharide that has been reported to increase the tolerance against water deficit in plants (Peterbauer et al., 2002; Peters et al., 2007). Evers et al. (2007) submitted potato clones (*Solanum tuberosum* L.) to a continuously increasing drought stress in a field trial and found differential accumulation of osmotically active solutes in both stressed genotypes, such as galactinol synthase that was significantly more expressed in drought-stressed plants. In *A. thaliana,* galactinol synthase isoform expression increased during drought and overexpression increased drought tolerance (Taji et al., 2002).

PPIase - Are ubiquitous proteins found in the cytosol of both prokaryotic and eukaryotic cells and in organelles such as ER, mitochondria and chloroplasts (Galat and

Metcalfe, 1995; Matouschek et al., 1995). Responses of PPIase to environmental changes have been reported. Sharma and Sing (2003) submitted young sorghum (*Sorghum bicolor* (L.) Moe.) to water stress and found that PPIase activity was induced in different tissues at various stages of development, in tolerant cultivar. As to authors, the differential effect of drought stress on PPIase activity in both cultivars was independent of water-potential, suggesting different regulatory pathways in the drought-tolerant and susceptible cultivars of sorghum.

3.2 Protein identified during 6d- water suppression in Senegal 55 437 by 2-DE SDS-PAGE

Taking in account that Senegal-55437 is a peanut reference to drought tolerance (Boote and Hammond, 1981; Azevedo Neto et al., 2010; Pereira et al., 2012; Duarte et al., 2013), and under water stress conditions, several up regulated protein are displayed (Jain et al., 2001; Duarte et al., 2011), we chose this genotype to further 2-DE assays.

Here, a total of 52 differentially expressed proteins were found, among them 32 were identified in water-stressed treatment, although not directly involved in response to drought tolerance. The subcellular location of the most proteins was Golgi complex, mitochondria, plastids and cytoplasm. Several proteins involved in redox homeostasis were identified, and also in metabolic functions such as photosynthesis, β-oxidation and osmoprotection. Table 1 displays the more representative proteins found in stressed peanut leaves, all of them are involved with responses to abiotic stress tolerance in several crops.

Kinesin-like protein KCA2 - Cytosolic free calcium (Ca^{2+}) is present in plant cells and its concentration depends on environmental signals, which are mediated directly by activating of calcium-dependent protein kinases or indirectly through Ca^{2+}-modulator proteins such as calmodulin (CaM)[1] and calmodulin-like proteins (Reddy et al., 1999; Tognetti et al., 2012). Ca^{2+}/CaM regulates a variety of unrelated target enzymes/proteins involved in various Ca^{2+}-mediated signal transduction pathways in plants, including those involved in cellular and physiological processes as diverse as cell division, ion transport, gene regulation and stress tolerance (Tognetti et al., 2012). The kinesin superfamily of microtubule motor proteins is comprised of conventional kinesin heavy chains and other related proteins called kinesin-like proteins that hydrolyze ATP and use the derived energy to translocate unidirectionally on microtubules (Pereira and Goldstein, 1994). Kinesins and kinesin-like proteins are

implicated in controlling diverse functions including spindle formation, chromosome segregation during cell division, and organellar and vesicular transport (Bloom and Endow, 1994; Vernos et al., 1995). Reddy et al. (1996) reported isolation of a cDNA encoding a novel kinesin-like calmodulin-binding protein (KCBP) from *Arabidopsis*. The calmodulin-binding motif was mapped to a short stretch of 23 amino acids in the carboxyl-terminal region of the protein. The predicted amino acid sequence showed significant sequence similarity with the motor domain of kinesin heavy chain and contains structural features associated with kinesins and kinesin-like proteins.

Fig. 2. 2-D SDS-PAGE profile of water stress-responsive proteins in leaves of peanut tolerant to drought (Senegal-55437). Proteins were separated over the pI range 4–7 at 12.5% SDS–PAGE.

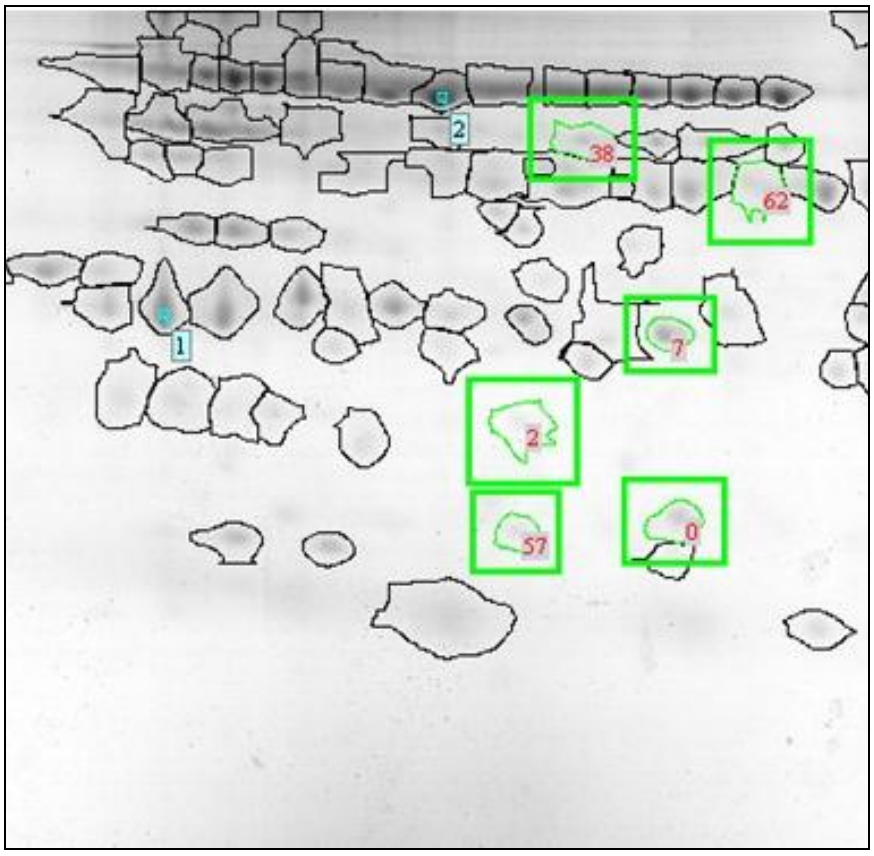

Table 1 Proteins identified in leaves from Senegal 55437 plants submitted to 6d-water suppression. Spot number, protein name, accession number, experimental mass and pI.

Spot nº	Protein	NCBI accession	Mass (Da)/pI
0.2	Kinesin-like protein KCA2	KCA2_ARATH	140880/5.91
2	Probable enoyl-CoA hydratase 1,	ECH1P_ARATH	28800/9.11
7	Photosystem I reaction center subunit II-2	PSAD2_ARATH	22350/9.78
38	AP2/ERF and B3 domain-containing transcription factor	RAVL2_ARATH	38855/ 6.62
57	ATP-dependent DNA helicase 2 subunit	KU80_ORYSJ	77964/ 7.53
62	Defensin-like protein 218	DF218_ARATH	10226/ 8.83

Enoyl-CoA hydratase (ECH) - This is one of enzymes involved in peroxisomal fatty acid β-oxidation. Peroxisomes are ubiquitous cytoplasmic organelles encased in a single lipid bilayer that contain hydrogen peroxide-producing oxidases and catalases to inactivate reactive molecules (Olsen, 1998; Zolman et al., 2001). In plants, peroxisomes also contain enzymes that act in photorespiration (Olsen, 1998) and the catabolism of branched-chain amino acids. Acording to (Zolman et al., 2011), *Arabidopsis* and other oilseed plants β-oxidize LCFAs in peroxisomes to provide energy during germination.

Very long chain fatty acids are important components of plant lipids, suberins, and cuticular waxes. Wax accumulation display an important function to limit water loss from plants grown in drought environment because it helps leaves in retention of water by minimizing cuticular transpiration (Jordan et al., 1984; Jefferson et al., 1989). Genotypes with low cuticular transpiration rates usually have a functional advantage during water deficit environments due to more efficient water use (Walker and Miller, 1986; Paje et al., 1988). Several reports have shown that drought stress can increase the amount of wax deposited on leaf surfaces in plants, and increased amounts of cuticular waxes are associated with drought tolerance in oat (Bengston et al., 1978), rice (Islam et al., 2009), sorghum (Jordan et al., 1984), peanut (Samdur et al., 2003), cotton (Bondada et al., 1996), among others. (Yang et al., 2011) investigated the relationship between

wax accumulation and drought tolerance in *Arabidopsis*. To accumulate cuticular waxes on the surface of *Arabidopsis* leaves, they used a transcription factor, WAX INDUCER1/SHINE 1, which regulates the expression of genes that control accumulation of cuticular wax. An ECR-based gene induction system was adopted to control gene expression. The authors found that induced expression of WIN1/SHN1 resulted in significant changes in cuticular wax biosynthesis, accumulation of very large amounts of cuticular waxes, enhanced drought tolerance, and reduced the number of stomata.

Photosystem I reaction center subunit II-2 - Oxygenic photosynthetic organisms contain two membrane-embedded photochemical reaction centers: Photosystem II (PSII) and Photosystem I (PSI). Each functions to move electrons against a potential gradient utilizing the energy captured by an excited chlorophyll. PSI1 extracts electrons from water and passes them through an electron transport chain to PSI, serving as the prime source of reducing power utilized by life forms for the production of carbohydrates, as well as the major source of atmospheric oxygen supporting respiration (Hall and Rao, 1987). When photosynthesis decreases, the excess excitation energy in PSII leads to an impairment of photosynthetic function and to accumulation of reactive oxygen species, thereby resulting in oxidative stress (Aranjuelo et al., 2011; Wilhelm and Selmar, 2011).

PS I and PS II are major stress perception systems in thylakoid membranes. PSII is the most important protein-pigment complex in the chloroplast that is also most vulnerable to drought stress (Baker, 2008). The efficiency of both in electron transport is declined under severe drought, due to limitation in CO_2 uptake coupled with an increased excitation energy in PSII and absorption of light energy in excess, leading to an imbalance between PSII activity and the Calvin cycle, with further consequences to whole chain between PSI and PSII (Carpentier, 1999; Baker, 2008).

Sperdouli and Moustakas (2012) studied the effect of mild, moderate and severe drought stress on PSII of 4-week-old *Arabidopsis thaliana* plants. The heterogeneity in all chlorophyl fluorescence parameters was maintained throughout water stress. According to authors, at end of drought stress, *Arabidopsis* leaves functioned normally under moderate drought stress, but the metabolic tolerance mechanisms under severe drought stress were limited, based on results obtained by lipid peroxidation. A decreased quantum yield for dissipation after down-regulation in PSII was also verified,

indicating that energy dissipation by down-regulation did not function and electron transport was depressed.

In peanut, Lauriano et al. (1997) submitted 11 w-genotypes (57-422, GC 8-35 and 73-30) to 16 d of total water suppression and found that photosynthetic capacity was decreased. The relative water content was reduced to 95 to 70 %. PSI and PSII electron transport activities decreased under drought, in, at least, two genotypes. All cultivars showed decreases in photochemical quenching (qP) and quantum yield of PS2 electron transport (Φe). According to authors, GC 8-35 was the most affected to water stress and 57-422 showed a higher degree of tolerance being gradually affected in photosynthetic capacity (PC) in contrast to the two other genotypes, which showed a sharp decrease in PC at the beginning of the drought cycle. This work provide interesting information for exploration of this genetic resource for further breeding program focused on dry environments.

AP2/ERF and B3 domain-containing transcription factor - belongs to a subfamily transcription factors specific of plants that share a conserved AP2/ERF DNA binding domain of 57–66 amino acids in size (Okamuro et al., 1997). The AP2/ERF genes constitute a large multigene family divided into four subfamilies named AP2, CBF/DREB, ERF, and RAV based on their sequence similarities and numbers of AP2/ERF domains (Sakuma et al., 2002). This last contain one AP2/ERF domain and a B3 domain, which are involved in distinct types of the transcription, such as biotic and abiotic stress responses (Sohn et al., 2006). Genome-wide expression analyses of AP2/ERF family genes in tomato (Sharma et al., 2010) and rice (Sharoni et al., 2011) reveal that many ERF subfamily genes are also induced by low or high temperature, dehydration or high salinity.

In soybean, Zhang et al. (2008) found a new member of the AP2/ERF transcription factor family, named *GmERF3, which expression* was induced by treatments with high salinity, drought, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). According to authors, *GmERF3* as an AP2/ERF transcription factor may play dual roles in response to biotic and abiotic stresses in plants.

In peanut, Na Chen et al. (2012) cloned full-length sequences of six genes from an ESTs- cDNA library, whose amino acid sequences contained AP2/ ERF domain. The expression patterns of the genes analyzed under cold, salt and drought stress. The results indicated that the expression of *AhERF4* and *AhERF6* were rapidly and substantially

enhanced under abiotic stress. The expression of *AhERF1* and *AhEERF5* were slightly enhanced under certain stress conditions. Some genes were down-regulated when under stress, such as *AhEERF3* in leaves under salt stress and *AhERF2* in leaves under drought stress. Authors suggest that different ERF proteins may have different functions in peanut abiotic stress acclimation.

ATP-dependent DNA helicase 2 subunit - Helicases are transcriptional activators commonly present with a function to unpacked nucleic acid particularly DNA, RNA or DNA–RNA hybrid through regulatory processes involving replication, transcription, translation, repair/recombination and ribosome biogenesis, and also involvement with abiotic stress tolerance in plants (Nakamura et al., 2004; Tuteja et al., 2012). Most of helicases belong to the DEAD box family with the presence of highly conserved residues Asp(D)-Glu(E)-Ala(A)-Asp(D) in motif II and contain nine motifs, also involved in translational initiation regulation (Tuteja et al., 2012).

Literature displays several articles reporting involvement of helicases in environmental stressed plants. Nakamura et al. (2004), performed modified differential display in order to isolate cDNAs corresponding to transcripts that accumulate preferentially in salt-stressed barley. A *HVD1* (*Hordeum vulgare* DEAD box protein) was characterized as a salt-responsive transcript, encoding a putative ATP-dependent RNA helicase. According to authors, *HVD1* mRNA was detected at low levels in leaves and roots of non-stressed plants, whereas its accumulation was transiently induced eight-fold higher under salt stress. The level of mRNA was increased by salt and cold conditions. HVD1 protein regulates the function of transcripts concerned with salt tolerance, or important metabolism such as photosynthesis, in chloroplasts.

In pea, Sanan-Mishra et al. (2005) reported the isolation of a DNA helicase 45 (PDH45) that is induced in seedlings in response to high salt, and exhibits striking homology with the eukaryotic translation initiation factor eIF-4A. The overexpression of *PDH45* in tobacco plants conferred salinity tolerance, providing a perspective of exploitation of DNA/RNA unwinding pathways for engineering salinity tolerance without affecting yield in crop plants.

In peanut, Majulatha et al. (2014) improved drought tolerance in plants by overexpressing a stress-responsive DNA *helicase, PDH45, from Pisum sativum L.* Several transgenic lines showed normal phenotype and increased chlorophyll stability under stress. Authors reported that transgenic lines showed 17.2 % and 26.75 %

increase in yield under non-stress and stress conditions over wild type ascertaining the feasibility of trait pyramiding strategy for the development of drought-tolerant peanut.

Defensin-like protein 218- Defensins are ubiquitous cationic peptides that belong to a large superfamily of antimicrobial peptides found in several organisms. Plant defensins are mainly localized to the cell wall and extracellular space of seeds (Terras et al., 1995) and also to the epidermis and vascular bundle of pea pods (Terras et al., 1995; Almeida et al., 2000). These proteins present numerous biological activities, such as inhibiting protein synthesis, ion channel function and amylase and trypsin activity; impairing microbial, root hair and parasitic plant growth; mediating abiotic stress and Zn tolerance; altering ascorbic acid redox state which can contribute to the activation of enzymatic defense APX (Terras et al., 1995; Almeida et al., 2000).

SA, MeJA, ABA and H_2O_2, have been reported to be involved in signal tranduction pathways for the activation of defense-related genes, including defensins (Hong and Hwang, 2002; Penninckx et al., 1996). The most articles of defensins available in literature has reported their holes in biotic stress, however, some articles reporting to defensin involvement in abiotic stresses have also been found. (Do et al., 2004) isolated various PR genes from a cDNA library constructed from poly(A)$^+$ mRNA from pepper (*Capsicum annuum* L.) leaves infected with *X. campestris* pv. *vesicatoria*. Several defense proteins were found. Among them, they cloned a defensin *CADEF1* cDNA whose hole was examined upon bacterial attack, wounding and water stress using northern blot analysis and in situ hybridization technique. The authors found strong accumulation of *CADEF1* mRNA in leaves in response to wounding, high salinity and drought stress, and suggest that bacterial pathogen infection, abiotic elicitors and some environmental stresses may play a significant role in signal transduction pathway for *CADEF1* gene expression.

In peanut, Govind et al. (2009) found nearly 700 genes in subtractive cDNA library from gradual process of drought stress adaptation. Fifty genes (25 regulators and 25 functional related genes) were selected for their stress responsiveness using northern blot analyses. The most abundant in library were involved in LEA proteins, heat shock proteins, proline rich protein and defensins, which are known for their stress responsive nature and confirmed their nature of differential regulation under different field capacity of drought stress treatments. Though defensins are majorly known to be upregulated in response to biotic stress, the authors noticed their induction under abiotic stress such as drought.

4. Conclusions

Water deficit is one of the most important abiotic stresses responsible for losses in productivity of several crops. The most of breeding program of legume and fibrous developed to semiarid environment have adopted strategies to sustainable use of water and extensive screening for drought tolerance in field conditions. In addition, biotechnological approaches have provided broad contributions in selection procedures, by using the current data generated by genomic and proteomic tolls.

In studies that focus on the identification of genes involved in environmental stresses, the certification of the status quo of the plant is fundamental so that the correct inferences can be made of the events associated with such processes.

Peanut is a short-cycle plant is widely known for its adaptation to tropical and semi-arid climate environments. In spite of this, genotypes respond differently when subjected to environmental stresses, depending on the period and intensity. The literature provides several reports focusing on response of peanut to drought tolerance, based on physiological, biochemical and molecular data, all of them have provided broad contribution to progress of classic improvement of crop.

In the present work, two contrasting genotypes were submitted to a short water stress in order to found proteins associated to drought tolerance, for further use as a molecular marker in assisted selection procedures of peanut breeding.

In whole, 52 proteins were identified, many of which were present in both control and stress treatments, indicating that their expressions were not directly involved with the central focus of this work, which is tolerance to water stress. In several articles available in the literature, we often find a list of all proteins identified through proteomic analysis. As our intention is to identify possible protein markers that may aid in selection procedures, we focus on only six directly involved with the proposed event (Table 1). These results may be useful as potential markers for molecular screening in order to identify drought tolerant genotypes, assisting the breeding of crop focused in semiarid environment.

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