UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

RODOLPHO GLAUBER GUEDES SILVA

RESPOSTAS FISIOLÓGICAS E BIOQUÍMICAS DO FEIJÃO-DE-CORDA [*Vigna unguiculata* (L.) Walp.] SUBMETIDO AO ESTRESSE HÍDRICO, INFECTADO COM O VÍRUS DO MOSAICO SEVERO DO CAUPI, E SOB PRESSÃO DOS DOIS ESTRESSES COMBINADOS

FORTALEZA
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Tese apresentada ao Programa de Pós-Graduação em Bioquímica, do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, como requisito parcial para obtenção do Título de Doutor em Bioquímica. Área de concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. José Tadeu Abreu de Oliveira.

FORTALEZA
2016

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca de Ciências e Tecnologia

B469a Silva, Rodolpho Glauber Guedes.
Respostas fisiológicas e bioquímicas do feijão-de-corda [*Vigna unguiculata* (L.) WALP.] submetido ao estresse hídrico, infectado com o vírus do mosaico severo do caupi, e sob pressão dos dois estresses combinados **/** Rodolpho Glauber Guedes Silva. – 2016.
137 f. : il., color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências, Departamento de Bioquímica e Biologia Molecular, Programa de Pós-Graduação em Bioquímica, Fortaleza, 2016.
Área de concentração: Bioquímica Vegetal.
Orientação: Prof. Dr. José Tadeu Abreu de Oliveira.

1. Estresse hídrico. 2. Feijão-caupi - Fitopatologia. 3. Vírus do mosaico do feijão. I. Título.

CDD 574.192

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Aprovada em: 14/04/2016

BANCA EXAMINADORA

Prof. Dr. José Tadeu Abreu de Oliveira
(Orientador)
Universidade Federal do Ceará (UFC)

Profa. Dra. Maria Izabel Florindo Guedes
Universidade Estadual do Ceará (UECE)

Prof. Dr. Joaquim Albenísio Gomes da Silveira
Universidade Federal do Ceará (UFC)

Prof. Dr. Maria Raquel Alcântara Miranda
Universidade Federal do Ceará (UFC)

Dr. Gilvan Pessoa Furtado
Fundação Oswaldo Cruz - CE

AGRADECIMENTOS

A Deus pela minha existência.

Aos meus pais, Rosangela Guedes e José Maria, pelo amor incondicional e por nunca terem medido esforços para que eu pudesse chegar até aqui. Serei eternamente grato!

A toda minha família.

Ao professor Tadeu por ser um grande exemplo de honestidade, responsabilidade, compromisso, inteligência, dedicação e por me fazer acreditar que ainda existem pessoas assim neste mundo. EXCELENTE orientador, tenho orgulho de você!

À professora Ilka pelo enorme carinho, por ser um grande exemplo de ser humano e por ter sido, indiscutivelmente, a melhor professora que tive durante toda minha vida!

A todos os professores do Departamento de Bioquímica e Biologia Molecular representados pelo Prof. Dr. Bruno Rocha que muito contribuíram para a minha formação intelectual e científica.

Ao Prof. Octavio Franco (Universidade de Brasília) e Kleber Souza pela parceria científica.

Aos professores Joaquim Albenísio, Raquel Miranda, Maria Izabel e Gilvan Pessoa por terem aceitado participar desta banca. Obrigado pela colaboração científica e disponibilidade.

Aos amigos que fazem ou já fizeram parte do Laboratório de Proteínas Vegetais de Defesa (Anna Lídia, Thiago, Handerson, Ivna, Larissa, Queilane, Ana Lu, Larissa, Aline, Iara, Hudson, Bárbara, Dhel, Pedro, Fredy, Louise,

Raissa, Jordania e Darcy) pela companhia, auxílio e momentos de descontração.

A todos do Laboratório de Tóxinas Vegetais que sempre estiveram disponíveis para ajudar nas pesquisas do nosso laboratório.

À Ana Karla pela amizade, por ter me auxiliado nos experimentos de fisiologia e pelos momentos de descontração.

À minha grande amiga Anna Lídia pela amizade de uma década, por me entender tão bem e sempre me mostrar que coisas que parecem tão complicadas podem se tornar simples resolver.

Ao meu grande amigo Marcos Sebastião por ser como um anjo na minha vida e nunca ter me abandonado nos momentos que precisei. Obrigado por me mostrar que o amor de uma amizade verdadeira não tem limites. Serei eternamente grato!!!

À Jasmine Castro e Clidia Moreira pela grande amizade! Vocês são dois dos maiores presentes que Fortaleza me deu! Amo muito!!! Obrigado pela atenção e companheirismo sempre demonstrados.

Ao Hermínio Jr pela amizade conquistada que já duram dez anos e por ser meu exemplo de inteligência e dedicação!

Ao Felipe Fernandes por ter me ensinado que posso, a cada dia, me tornar uma pessoa melhor, pelo apoio, amor e por ter me dado bens tão preciosos: Amora, Cookie e Esperança (nossos cachorros). Através deles, conheci a importância da adoção e o quanto amo animais! Não tenho palavras pra dizer o quanto sou grato!

Por fim, agradeço a todos que contribuíram direta ou indiretamente para a realização deste trabalho.

AGRADECIMENTOS INSTITUCIONAIS

Este trabalho foi realizado graças ao auxílio das seguintes Instituições:

Departamento de Bioquímica e Biologia Molecular do Centro de Ciências da Universidade Federal do Ceará (**DBBM-UFC**).

Coordenação de Aperfeiçoamento de Pessoal e de Ensino Superior (**CAPES**), pela bolsa de Pós-Graduação concedida ao autor, através de convênio com o Programa de Pós-Graduação em Bioquímica do Departamento de Bioquímica e Biologia Molecular do Centro de Ciências da Universidade Federal do Ceará.

Conselho Nacional de Desenvolvimento Científico e Tecnológico (**CNPq**), através de convênio com o Programa de Pós-Graduação em Bioquímica do Departamento de Bioquímica e Biologia Molecular do Centro de Ciências da Universidade Federal do Ceará.

Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (**FUNCAP**), através de convênio com o Programa de Pós-Graduação em Bioquímica do Departamento de Bioquímica e Biologia Molecular do Centro de Ciências da Universidade Federal do Ceará.

RESUMO GERAL

O feijão-de-corda (*Vigna unguiculata*) é uma das principais leguminosas cultivadas no Brasil. Entretanto, devido à presença de fatores bióticos e abióticos são geradas perdas significativas na produtividade desta cultura. O *Vírus do Mosaico Severo do Caupi* (CPSMV) tem atacado com grande frequência os cultivos brasileiros. Além disso, devido o feijão-de-corda ser cultivado em regiões onde os índices pluviométricos são baixos, a deficiência hídrica é bastante comum. Esse estresse promove impacto negativo substancial no crescimento e desenvolvimento das plantas, resultando em diminuição na produção de biomassa. Em condições de campo, os estresses mencionados acima podem ocorrer tanto individualmente quanto simultaneamente. Portanto, o presente estudo teve como objetivo estudar as respostas do feijão-de-corda (genótipo CE-31, sin. Pitiúba) submetido ao estresse hídrico, infectado com o CPSMV e sob pressão dos dois estresses combinados. Sob essas condições, a cinética enzimática de proteínas antioxidantes (superóxido dismutase, catalase e ascorbato peroxidase) e PR-proteínas (glucanase, quitinase e guaiacol peroxidase), bem como a dosagem de peróxido de hidrogênio e quantificação do CPSMV em folhas de feijão-de-corda foram realizadas. Além disso, proteínas diferencialmente acumuladas em folhas de feijão-de-corda submetidas à esses estresses foram quantificadas e identificadas por LC-MS/MS e bancos de dados disponíveis. A infecção viral não alterou a maioria dos parâmetros fisiológicos. Já a deficiência hídrica contribuiu para a diminuição da fotossíntese líquida, transpiração e condutância estomática. Até 6 dias após inoculação do CPSMV, o vírus não agravou os efeitos negativos da seca. Os resultados da cinética enzimática revelaram que as respostas são alteradas de formas diferentes frente aos estresses quando impostos individualmente ou simultaneamente e que a presença do estresse hídrico favoreceu a infecção viral após 2 dias de inoculação. A análise proteômica revelou 117 proteínas diferencialmente acumuladas pertencentes a diferentes categorias de acordo com as funções fisiológicas: energia e metabolismo, fotossíntese, metabolismo de proteínas, homeostase redox, fatores de regulação e processamento de RNA, resposta ao estresse, defesa de plantas e outras. Estes resultados são importantes para a compreensão dos

mecanismos celulares que atuam no feijão-de-corda, genótipo CE-31, quando submetido ao estresse hídrico, à infecção pelo CPSMV e a ambos os estresses de forma combinada.

Palavras-chave: Feijão-de-corda, deficiência hídrica, CPSMV, estresses combinados

ABSTRACT

Cowpea (*Vigna unguiculata*) is a legume (*Fabaceae*) with high nutritional value grown in Brazil. However, biotic and abiotic factors are responsible for causing significant losses to the growth and yield of leguminous plants. Cowpea mosaic severe virus (CPSMV) is one of the more severe problems affecting cowpea production in Brazil. Also, cowpea are mainly grown in regions where rainfall is low, thus water deficit is quite common. This stress promotes substantial negative impact on growth and development of plants, resulting in decreased of biomass production. Under field conditions, the stresses mentioned above can occur both individually and simultaneously. Therefore, this work aimed to study the responses of cowpea (CE-31 genotype, syn. Pitiuba) subjected to water deficit, infected with CPSMV and under pressure of the two combined stresses. Under these conditions, enzyme kinetics of antioxidant protein (superoxide dismutase, catalase and ascorbate peroxidase) and PR-proteins (glucanase, chitinase and guaiacol peroxidase) as well as hydrogen peroxide dosage and quantification of CPSMV in cowpea leaves were performed. In addition, differentially accumulated proteins from cowpea leaves subjected to these stresses were identified and quantified by LC-MS/MS. Viral infection did not affect the majority of physiological parameters. Already water deficit contributed to the decrease in net photosynthesis, transpiration and stomatal conductance. At 6 days after CPSMV inoculation, the virus did not worsen the negative effects of drought. The results revealed that the enzyme kinetics of the responses was changed across different ways when the stress imposed individually or simultaneously and the presence of water deficit favored viral infection after 2 days of CPSMV inoculation. The proteomic analysis revealed 117 differentially accumulated proteins belonging to different categories according to the physiological functions: energy and metabolism, photosynthesis, protein metabolism, redox homeostasis, regulation factors and RNA processing, response to stress, plant defense and others. These results are important for understanding the cellular mechanisms that operate in cowpea (CE-31 genotype), when subjected to water deficit, CPSMV infection and both combined stresses.

Keywords: Cowpea, water deficit, CPSMV, combined stresses

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LISTA DE ABREVIATURAS E SIGLAS

APX	Ascorbato peroxidase
Avr	Avirulencia
CAT	Catalase
CHI	Quitinase
C_i	Concentração interna de CO_2
CMV	Vírus do mosaico do pepino
CPSMV	Virus do mosaico severo do caupi
E	Taxa de transpiração
eEF-1A	Fator de elongação da tradução 1ª
ETI	Imunidade desencadeada por efetores
ETR	Taxa de transporte de elétrons
Fv/Fm	Eficiência quântica potencial do fotossistema II
GLU	Glucanase
g_s	Condutância estomática
HR	Resposta hipersensitiva
HSP	Proteína de choque térmico
LVM	Vírus do mosaico do alface
MAMPS	Padrões moleculares associados aos microrganismos
miRNA	MicroRNA
OEE1	Proteína 1 do complexo de evolução do oxigênio
PAMPS	Padrões moleculares associados aos patógenos
PMMoV	Vírus do mosqueado do pimentão
P_N	Fotossíntese líquida
P_N/C_i	Eficiência instantânea de carboxilação
P_N/E	Eficiência do uso da água
POX	Peroxidase do guaiacol
PPFD	Densidade do fluxo de fótons fotossintéticos
PRR	Receptores de reconhecimento de padrões
Prx	Peroxirredoxina
PSI	Fotossistema I
PSII	Fotossistema II

PTGS	Silenciamento gênico pós-transcricional
PTI	Imunidade desencadeada por PAMPs
RISC	Complexo de silenciamento induzido por RNA
RNAi	RNA de interferência
ROS	Espécies reativas de oxigênio
siRNA	Pequeno RNA de interferência
SOD	Superóxido dismutase
sRNAs	Pequenos RNAs
TGS	Silenciamento gênico transcricional
TMV	Vírus do mosaico do tabaco
TYMV	Vírus do mosaico amarelo do nabo
VPg	Proteína viral ligada ao genoma
ΔF/Fm’	Eficiência quântica efetiva do fotossistema II

SUMÁRIO

CAPÍTULO I

Fundamentação teórica

Rodolpho Glauber Guedes Silva

1 FUNDAMENTAÇÃO TEÓRICA

1.1 Feijão-de-corda

O feijão-de-corda (FIGURA 1), também conhecido como feijão miúdo, feijão-de-corda, feijão fradinho, feijão frade ou feijão macassar (*Vigna unguiculata* [L.] Walp.) é uma leguminosa (*Fabaceae*) de alto valor nutricional cultivada principalmente na Ásia, África e América Latina, abrangendo mais de 10 milhões de hectares (NEDUMARAN, ABINAYA, SHRAAVYA, RAO, E BANTILAN, 2013). Estima-se uma produção anual de mais de 4,5 milhões de toneladas de grãos (SINGH et al., 2003). Suas sementes apresentam alto conteúdo de proteínas (23%), carboidratos (56%) e fibras (4%), sendo bastante utilizadas como fonte de alimentação (IQBAL et al., 2006). Além disso, suas vagens verdes e folhas são usadas como vegetais verdes e as partes secas como ração animal.

FIGURA 1 - Representação de plantas de feijão-de-corda com 14 dias de crescimento em casa de vegetação.

Fonte: próprio autor.

O feijão-de-corda é cultivado em todas as regiões do país, mas predomina nas regiões Norte e Nordeste. No Nordeste, a produção de grãos em 2014 foi de 674.659 t e a produtividade alcançou 438 kg/ha. A Bahia (357.311 t), Ceará (109.148 t), Piauí (55.278 t), e Pernambuco (52.787 t) são os maiores produtores e apresentam as maiores áreas plantadas (IBGE, 2015).

Dentre os cultivares de feijão-de-corda, o CE-31 (Pitiúba), desenvolvido pela Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA-Meio Norte), apresenta tolerância e boa adaptação a diversos estresses (DANTAS et al., 2005; LOBATO et al., 2009; PRAXEDES et al., 2010; 2014). Entretanto, ainda existem vários fatores abióticos e bióticos, que reduzem, significativamente, a produção do feijão-de-corda como, por exemplo, estresse hídrico e infecções virais.

1.2 Estresses abióticos

As plantas são frequentemente expostas a diversos estresses abióticos como frio, calor, deficiência hídrica e salinidade. Esses estresses normalmente causam desequilíbrio na homeostase celular levando a uma série de alterações morfológicas, fisiológicas e moleculares (VAN VELTHUIZEN et al., 2007) que resultam em perdas que podem ultrapassar 50% para a maioria das culturas da agricultura mundial (WANG; VINOCUR; ALTMAN, 2003).

Diversos genes são induzidos ou reprimidos em consequência dos estresses abióticos (DELANO-FRIER et al., 2011; GRATIVOL; HEMERLY; FERREIRA, 2012). Dessa forma, os produtos desses genes podem atuar em resposta ao estresse no ambiente celular, como por exemplo, proteínas envolvidas na biossíntese de compostos osmoprotetores, sistemas enzimáticos antioxidantes, proteases, transportadores e chaperonas. Além disso, a ativação de proteínas regulatórias (fatores de transcrição, fosfatases e cinases) e moléculas de sinalização (como por exemplo, as espécies reativas de oxigênio - EROs) são extremamente importantes na regulação da transdução de sinal e expressão de genes responsivos ao estresse (WANG et al., 2009; PELEG, APSE; BLUMWALD, 2011). Assim, todas as tentativas de resposta buscam reestabelecer a homeostase celular.

Devido às mudanças climáticas, a deficiência hídrica tem se tornado cada vez mais severa e frequente, sendo um problema de grande importância mundial (BRESHEARS et al., 2005). Portanto, a deficiência hídrica consiste em um dos principais estresses abióticos que limitam a produtividade de muitas culturas importantes, como o feijão-de-corda.

1.2.1 Deficiência hídrica

As respostas ao estresse hídrico em plantas compreendem várias vias importantes, começando pela percepção do estresse até a expressão e/ou repressão de genes importantes (reprogramação genética), alterando a composição do transcriptoma, proteoma e metaboloma.

Um dos principais efeitos da seca é a diminuição na fotossíntese. Esse estresse provoca mudanças em pigmentos e componentes fotossintéticos (ANJUM et al., 2003), danifica o aparato fotossintético (FU; HUANG, 2001) e diminui atividades de enzimas do ciclo de Calvin (MONAKHOVA; CHERNYADÈV, 2002). Outro efeito importante que inibe o crescimento e a capacidade fotossintética das plantas é o desequilíbrio entre a produção de EROs e as defesas antioxidantes (FU; HUANG, 2001; REDDY, CHAITANYA E VIVEKANANDAN, 2004) que geram acúmulo de EROs altamente reativos, causando danos oxidativos a proteínas, lipídeos de membrana e outros componentes celulares. Além disso, durante a deficiência hídrica, para evitar a perda de água por transpiração, as plantas fecham os estômatos (MANSFIELD; ATKINSON, 1990), contribuindo com a diminuição da capacidade fotossintética. Os efeitos mencionados acima resultam na diminuição da expansão foliar, danos na maquinaria fotossintética, senescência foliar prematura e consequente diminuição na produtividade das culturas (WAHID; RASUL, 2005). Assim, os sistemas antioxidantes enzimáticos (tais como, superóxido dismutase, catalase, peroxidase, peroxidase do ascorbato e glutationa redutase) e não enzimáticos (tais como, cisteína, glutationa reduzida e ácido ascórbico) são extremamente importantes para diminuir os efeitos deletérios das EROs nas células vegetais (GONG et al., 2005).

Algumas plantas são capazes de ajustar o metabolismo e vias regulatórias para proteger as células dos efeitos danosos do estresse (KOSOVÁ et al., 2011). As plantas podem evitar o estresse hídrico acelerando seu ciclo de vida (florescendo nos estágios iniciais de vida, reduzindo a perda de água e aumentando sua captação por meio de alterações morfológicas) (KOH et al., 2015). Além disso, algumas plantas apresentam mecanismos de tolerância à deficiência hídrica alterando processos fisiológicos como a fotossíntese, respiração, relações hídricas e ajuste osmótico (LAWLOR; TEZARA, 2009; REDDY; CHAITANYA; VIVEKANANDAN, 2004).

1.3 Defesa de plantas contra patógenos

A interação planta-patógeno pode ser classificada em dois tipos: compatível, na qual o patógeno tem sucesso na infecção, e incompatível, na qual o patógeno não é capaz de se desenvolver no hospedeiro.

As plantas são constantemente desafiadas por patógenos como vírus, bactérias, fungos e herbívoros como nematoides e insetos. Os mecanismos de defesa contra esses organismos envolvem, basicamente, dois tipos de respostas: **barreiras constitutivas,** químicas e/ou físicas pré-formadas (cutícula, parede celular e fitoanticipinas) que limitam o acesso dos organismos às células vegetais (VLOT et al. 2009; THOMMA et al. 1998) e **respostas de defesa induzidas,** que envolvem o reconhecimento do patógeno pelo hospedeiros e a indução de uma série de sinais que resultam em respostas rápidas de defesa (defesa inata ou basal).

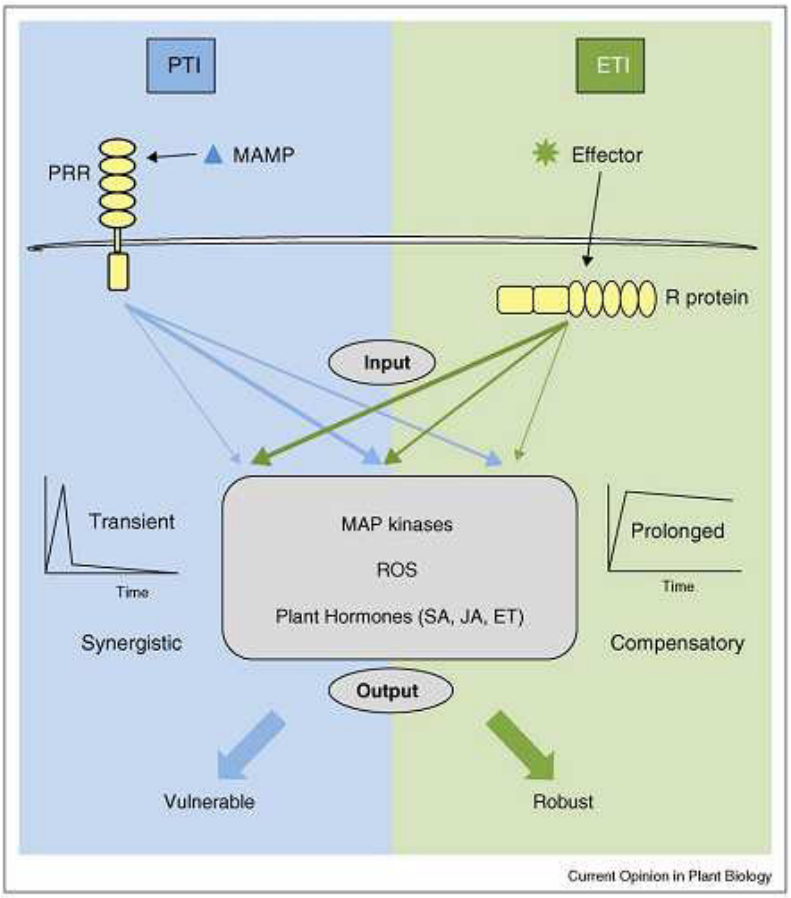
Com relação às defesas induzidas, as interações entre plantas e patógenos envolvem dois tipos de comunicação. Não só a planta é capaz de reconhecer e se defender contra um patógeno potencial, mas o agente patogênico também deve ser capaz de manipular a biologia da planta para criar um ambiente adequado para o seu crescimento e reprodução. Assim, tanto o patógeno quanto a planta têm evoluído um conjunto de genes que permitem essa interação. Segundo Jones e Dangl (2006), as repostas de defesa de plantas ocorrem de duas maneiras. Uma utiliza PRRs (*pattern recognition receptors*) que respondem a MAMPs (*microbial-associated molecular patterns*) ou PAMPs (*pathogen-associated molecular patterns)*, que ao interagirem com

P(M)AMPs desencadeiam uma cascata de respostas de defesa conhecidas, conjuntamente, como PTI (*PAMP-triggered immunity*) (ZIPFEL; FELIX, 2005). No entanto, o patógeno, com a evolução, foi capaz de suprimir os diferentes componentes da PTI por meio de proteínas efetoras. Nesse caso, uma segunda linha de defesa das plantas evoluiu, na qual genes R são expressos. Esses genes codificam proteínas R, agrupadas na família NBS-LRR (do Inglês, *Nucleotide Binding Site-Leucine Reach Repeats*), que interagem, direta ou indiretamente, com esses efetores dos patógenos, ou seja, com as proteínas de avirulência (Avr) do patógeno. Esse reconhecimento desencadeia uma resposta de resistência mais forte, ainda do que a basal, conhecida como ETI (do Inglês, *Effector-Triggered Immunity*) (DANGL; JONES, 2001). A FIGURA 2 resume o conjunto de repostas desencadeadas na PTI e ETI. Essa segunda linha de defesa (ETI) foi sugerida por Flor (1971) na forma de uma hipótese denominada gene-a-gene (FIGURA 3A).

Com o avanço dos estudos nessa área de pesquisa variações dessa hipótese gene-a-gene foram propostas, a exemplo daquelas conhecidas como modelo guarda e o modelo "*decoy".* No modelo guarda, os produtos dos genes R da planta atuam como proteínas guardiãs (do inglês, *guard protein*) e interagem, diretamente, com proteínas guardadas (do inglês, *guardee protein*), que são as proteínas alvos do patógeno. A interação de proteínas efetoras do patógeno com proteínas guardadas do hospedeiro resulta em uma modificação nesta molécula que se liga às proteínas guardiãs. Assim, as proteínas guardiãs sofrem modificações estruturais importantes para o desencadeamento da resposta de defesa (FIGURA 3B e C) (VAN DER BIEZEN; JONES, 1998; DANGL; JONES, 2001).

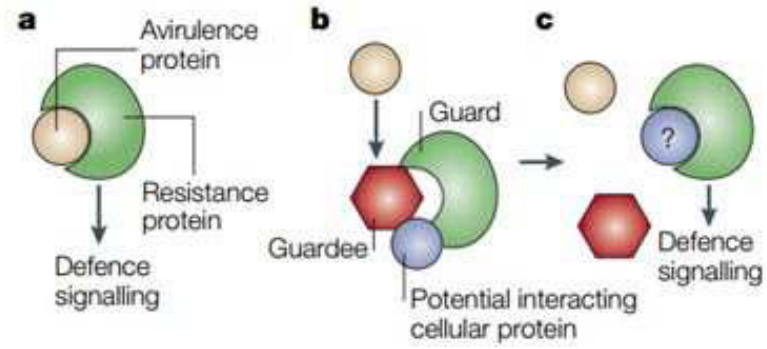
No modelo *"decoy"* existem proteínas no hospedeiro que atuam como iscas na percepção de efetores dos patógenos. Entretanto, por si só, essas proteínas não contribuem para a suscetibilidade ou resistência a doenças, diferente do modelo da hipótese guarda. Dessa forma, a proteína isca mimetiza alvos dos efetores do patógeno e são requeridas pelas proteínas-R para ativação da defesa do hospedeiro, funcionando como uma armadilha para induzir os eventos de reconhecimento. No entanto, na ausência de proteínas-R, as proteínas iscas não contribuem para desenvolvimento da doença (VAN DER HOORN; KAMOUN, 2009).

FIGURA 2 – Representação esquemática das respostas de defesa de plantas envolvendo ETI e PTI. M(P)AMP é reconhecido por uma PRR desencadeando a PTI. Um efetor é reconhecido por uma proteína de R para desencadear respostas correspondentes à ETI. Embora a maquinaria de sinalização empregada em PTI e ETI seja extensivamente compartilhada, ela é utilizada de forma diferente em ambas as repostas.



Fonte: TSUDA; KATAGIRI, 2010.

FIGURA 3 – Representação esquemática da hipótese gene-a-gene e hipótese guarda. (A) A hipótese prediz que as proteínas de resistência do hospedeiro interagem com proteínas de avirulência do patógeno, induzindo respostas de defesa. (B) Na hipótese de guarda, proteínas guardadas (guardees) são alvos das proteínas avirulência e são exigidas para a infecção bem sucedida pelo patógeno. Proteínas guardiãs (guard) e proteínas guardadas (guardee) interagem dinamicamente, podendo ter outra (s) proteína (s) no complexo. (C) Após a modificação da proteína guardada pela proteína de avirulência, a interação entre as proteínas guardiãs e guardadas é alterada e a guardiã desencadeia uma cascata de sinalização que conduz a defesa.



Fonte: SOOSAAR et al., 2005.

1.3.1 Defesa de plantas contra vírus

Vírus são pequenos parasitas intracelulares obrigatórios, que necessitam da maquinaria celular do hospedeiro para se multiplicar e colonizá-lo. O genoma viral consiste de uma fita de DNA ou RNA (simples ou dupla) que é protegido dentro de uma estrutura proteica denominada de **capsídeo**. A estrutura viral completa é chamada de **virion**.

Devido à presença de barreiras físicas pré-formadas nas plantas (por exemplo, a cutícula e parede celular), a infecção viral ocorre por meio de injúrias na planta ocasionadas por vetores como nematoides e insetos (ALEXANDER; CILIA, 2015) e por danos físicos nos seus tecidos. A consequência da interação planta-vírus depende do sucesso dos mecanismos de defesa da planta e da capacidade do vírus em revidar estas respostas

através do uso de fatores do hospedeiro para o seu próprio benefício (PALUKAITIS et al., 2013).

Os principais mecanismos de defesa de plantas contra vírus conhecidos são: resistência dominante, resistência recessiva e resistência mediada por RNAi.

1.3.1.1 Resistência dominante

A resistência dominante ocorre, basicamente, quando há reconhecimento dos produtos de genes de avirulência (avr) do patógeno por produtos de genes de resistência (R) do hospedeiro – é a interação gene-a-gene. Os produtos dos genes R normalmente são proteínas pertencentes à classe NBS-LRR (*nucleotide binding siteleucine-rich repeat*), já referidas anteriormente nessa seção. Além desse tipo de reconhecimento, os produtos R e avr podem interagir como nos modelos guarda e "decoy" (VAN DER BIEZEN; JONES, 1998).

1.3.1.2 Resistência recessiva

A resistência recessiva ocorre devido a mutações ou ausência de fatores no hospedeiro que são requeridos para a replicação viral. Um grande número de genes recessivos tem sido clonado de espécies vegetais e codificam fatores de iniciação eucarióticos (do inglês, *eukaryotic initiation factors* – eIFs) pertencentes às famílias 4E (eIF4E) e 4G (eIF4G) que juntos formam o complexo eIF4F. A duas isoformas desses fatores (eIF(iso)4E e eIF(iso)4G formam o complexo eIF(iso)4F. A principal função desse complexo é a ligação ao capacete do mRNA maduro da planta que é fundamental no processo de tradução (TRUNIGER; ARANDA, 2009; WANG; KRISHNASWAMY, 2012; JULIO *et al.*, 2014; REVERS; NICAISE, 2014). Assim, mutações nesses fatores, de um modo geral, impedem o reconhecimento dos ribossomos por proteínas virais (VPg), inibindo a síntese de proteínas. Por exemplo, Nicaise et al. (2003) verificaram que a substituição de um aminoácido (alanina para prolina) na sequência do fator eIF4E resultou na resistência do alface contra o LVM (*Lettuce Mosaic Virus*).

1.3.1.3 Resistência mediada por RNAi

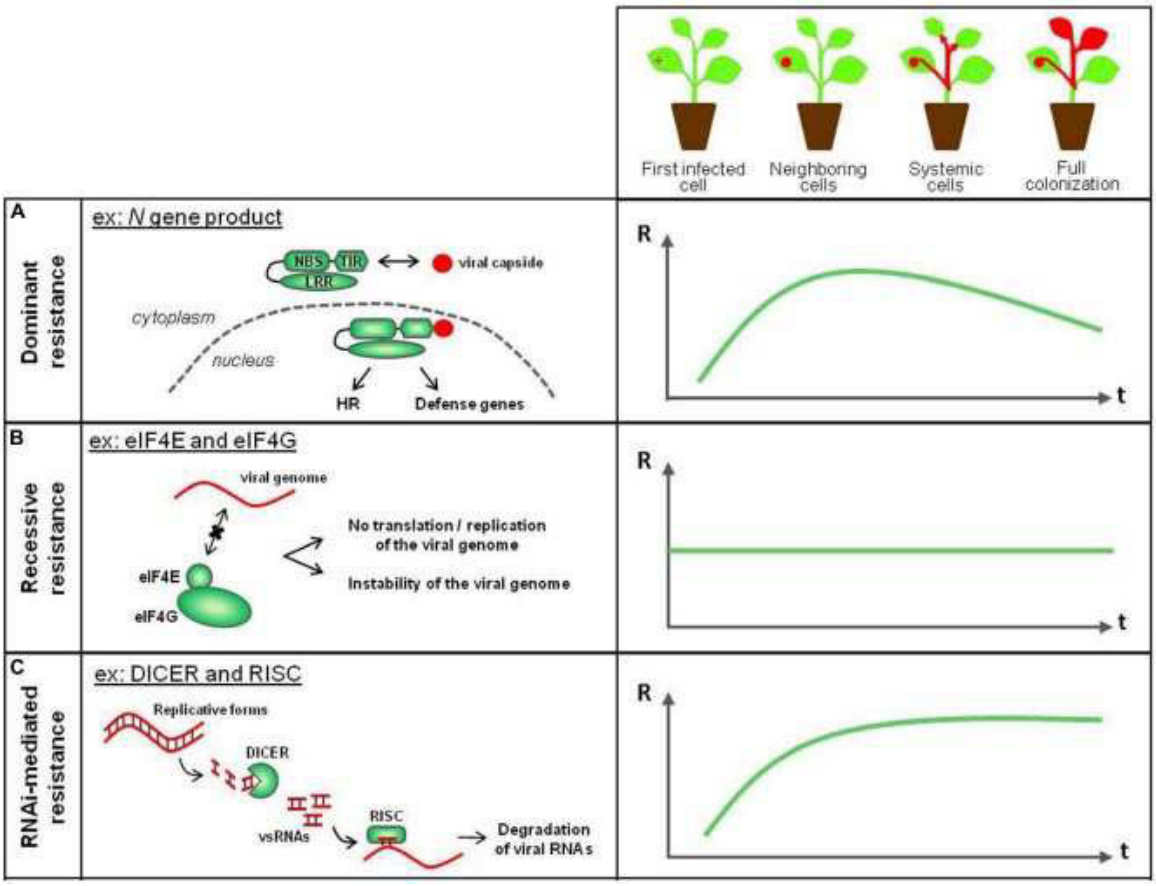
RNA de interferência (RNAi) corresponde a um processo celular responsável pelo silenciamento gênico pós-transcricional (PTGS, do inglês, Post-Transcriptional Gene Silencing) ou silenciamento gênico transcricional (TGS, do inglês, Transcriptional Gene Silencing), evolutivamente conservado na maioria das células eucarióticas, desencadeado por sRNAs (do inglês, *Small RNAs*) que são pequenas moléculas de RNA não codificantes (cerca de 20-30 nucleotídeos de comprimento). RNAi corresponde a um mecanismo específico da expressão gênica, tendo como alvos os ácidos nucleicos virais (PUMPLIN; VOINNET, 2013; SHUKLA; DALAL; MALATHI, 2013).

Os sRNAs podem ser classificados em miRNA (do inglês, *microRNA*) ou siRNA (do inglês, *Small interfering RNA*) de acordo com seu precursor e via de biogênese. Os miRNAs (21 a 24 nucleotídeos) são originados a partir de moléculas de RNA com emparelhamento de bases imperfeitos, resultando em estruturas em forma de grampos. Já os siRNAs são produzidos a partir de dsRNAs (do inglês, *double-stranded RNAs*), e requerem a atividade da enzima RNA polimerase RNA-dependente (KATIYAR-AGARWAL; JIN, 2010).

As moléculas de siRNA e miRNA podem induzir PTGS através da clivagem ou inibição da tradução pelo complexo ribonucleoproteico de silenciamento (RISC, do inglês: *RNA-induced Silencing Complex*). O componente catalítico do complexo RISC corresponde a um membro da família de proteínas Argonauta (AGO) que possui função de RNase (BOLOGNA; VOINNET, 2014). Já TGS regula a expressão gênica por meio da metilação do DNA e histonas (PUMPLIN; VOINNET, 2013).

A FIGURA 4 resume os três principais tipos de resistência contra vírus.

FIGURA 4 – Representação esquemática dos principais tipos de resistência contra vírus de plantas. (A) A resistência dominante baseia-se na interação entre um fator de virulência e um produto do gene R específico, e é eficaz vários dias após a entrada do vírus na planta. O fenômeno associado a HR restringe o patógeno nas células infectadas e vizinhas. (B) Resistência recessiva, que corresponde a ausência de fatores do hospedeiro que são necessários para a replicação viral é uma resistência não-induzível, passiva e eficaz ao longo a colonização da planta. (C) O RNA de interferência (RNAi) tem como alvo os ácidos nucleicos virais. Uma vez configurado, após alguns dias, a eficácia deste mecanismo de defesa aumenta e se espalha por toda a planta.



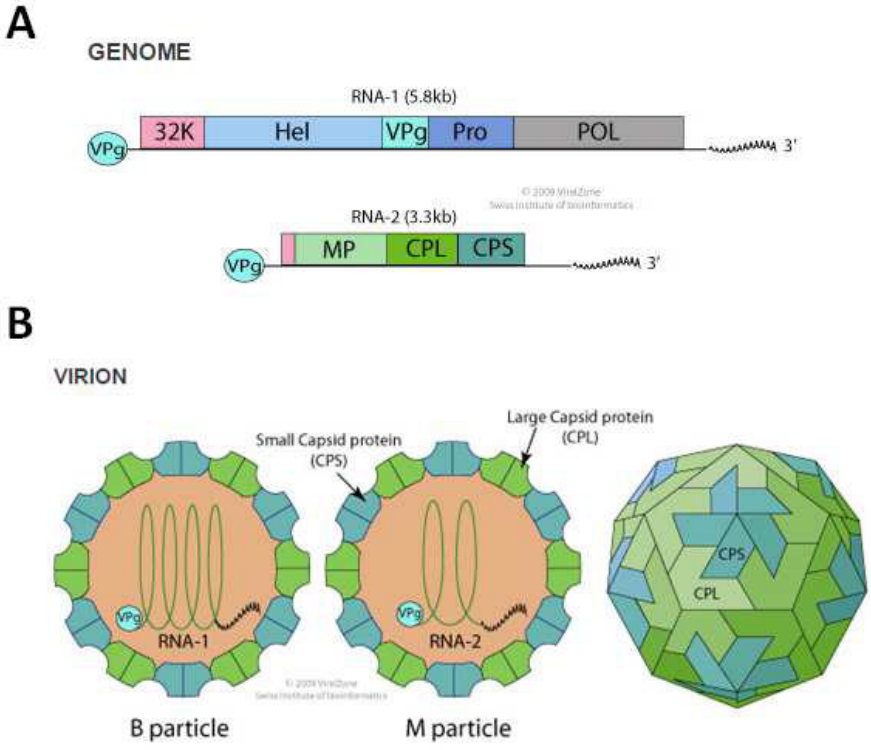
Fonte: NICAISE, 2014.

1.3.2 Cowpea severe mosaic virus (CPSMV)

O vírus do mosaico severo do feijão-de-corda (CPSMV, do Inglês: *Cowpea Mosaic Severe Virus*) é um dos vírus mais importantes que infecta o

essa cultura no Brasil (BOOKER et al., 2005; LIMA et al., 2005a). O CPSMV é um vírus isométrico (diâmetro entre 28 e 30 nm) pertencente à família *Comoviridae* e gênero *Comovirus*, com capsídeo constituído por 60 cópias de uma proteína maior (43 kDa) e 60 cópias de uma proteína menor (23 kDa). Seu genoma possui duas moléculas de RNA positivo de fita simples (RNA-1 e RNA-2). Essas moléculas de RNA possuem uma proteína conhecida como VPg (do Inglês: *Viral Protein Genome-linked*), ligada na extremidade 5' e uma cauda poli(A) na extremidade 3' (CHEN; BRUENING, 1992) (FIGURA 5). A molécula de RNA-1 codifica para proteínas necessárias para a replicação viral, enquanto RNA-2 codifica para duas proteínas do capsídeo e uma proteína envolvida no movimento célula-a-célula (LOMONOSSOFF; GHABRIAL, 2001; LE GALL; IWANAMI; KARASEV, 2005).

FIGURA 5 - Representação esquemática do genoma bipartido (A) e capsídeo do CPSMV (B). 32K - proteinase cofactor; Hel – helicase; VPg – proteína viral ligada ao genoma; Pro – proteinase; POL – polimerase; MP – proteína de moviemento; CPL – proteína do capsídeo grande; CPS – proteína do capsídeo pequena.



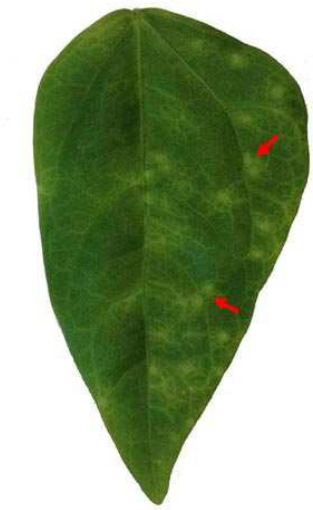
Fonte: ViralZone, 2016.

A replicação do genoma do CPSMV ocorre de acordo como o mesmo padrão de outros retrovírus com RNA de sentido positivo. Logo após a infecção da planta, o genoma viral (RNAs) é liberado do capsídeo e replicado em uma fita complementar de sentido negativo. Simultaneamente, a RNA dependente de RNA polimerase (RdRP), utiliza a fita complementar para produzir inúmeras cópias de RNA sentido positivo (LABIERTÉ; SANFAÇON, 2010).

Na natureza, a transmissão do CPSMV ocorre através de vetores (insetos da família *Chrysomelidae*), entre eles *Ceratomia arcuata*, o vetor mais importante no Brasil (LIMA *et al.*, 2005b). A taxa de transmissão pode ser variável, dependendo de vários fatores como: isolado viral, da espécie de coleóptero e condições ambientais (FULTON; SCOTT; GAMEZ, 1980; MATTHEWS, 1991; GERGERICH; SCOTT, 1996).

Os principais sintomas da infecção pelo CPSMV são: lesões cloróticas que caracterizam a formação do mosaico, má formação e desenvolvimento de bolhas em folhas jovens e diminuição do tamanho da folha (DE JAGER, 1979) (FIGURA 6).

FIGURA 6 – Imagem representativa de folha de feijão-de-corda infectada com CPSMV. As setas vermelhas apontam para as lesões necróticas que caracterizam a formação do mosaico.



Fonte: próprio autor.

O primeiro caso de infecção do feijão-de-corda pelo CPSMV no Brasil ocorreu no ano de 1947 no Rio Grande do Sul (OLIVEIRA; 1947) e, desde então, alcançou todas as regiões produtoras de feijão-de-corda do país (LIMA et al., 2005a, CAMARÇO et al., 2009; FREITAS et al., 2012). No Brasil, além de *Vigna unguiculata*, as principais espécies de plantas que são naturalmente infectadas pelo CPSMV são: *Glycine max*, *Phaseolus vulgaris*, *Macroptilium lathyroides*, *Canavalia brasiliensis*, *Canavalia ensiformis*, *Psophocarpus tetragonologus* e *Crotalaria juncea*, *Crotalaria paulinea* (BRIOSO et al., 1994; BERTACINI et al., 1998; LIMA et al., 2005b).

1.4 Estresses combinados

As plantas estão diariamente expostas a estresses bióticos e abióticos que podem ocorrer de forma isolada ou combinada. Assim, as plantas necessitam de respostas de defesa para garantir a sobrevivência das células frente aos danos provocados por esses estresses (SHAO et al., 2007). Uma das principais respostas de adaptação a essas condições é a modulação da expressão gênica. A especificidade da resposta é controlada por uma série de vias regulatórias que envolvem fatores de transcrição, EROs, fatores de choque térmico e pequenos RNAs que podem interagir uns com os outros (ATKINSON; URWIN, 2012).

Apesar da necessidade de compreender a tolerância aos estresses combinados em plantas, não há muitos estudos realizados nesse sentido. O conhecimento disponível, atualmente, indica que a ocorrência simultânea de estresses bióticos e abióticos podem causar efeitos negativos (ou seja, a suscetibilidade) ou positivo (isto é, a tolerância) em plantas, dependendo do estresse e patógeno em estudo (TIPPMANN; SCHLÜTER; COLLINGE, 2006). Por exemplo, plantas de feijão comum (*Phaseolus vulgaris*) submetidas ao estresse hídrico e infectadas com o fungo *Macrophomina phaseolina* apresentaram altas taxas de transpiração e temperatura foliar comparadas aos controles (plantas submetidas apenas ao estresse hídrico) (MAYEK-PEREZ *et al.*, 2002). Portanto, o estresse abiótico tornou a planta vulnerável à infecção pelo fungo. Da mesma forma, plantas de álamo híbrido (*Populus nigra* x *P. maximowiczii*) em condições de seca e infectadas com *Septoria musiva*

apresentaram cancros maiores (sintoma da doença causada por este patógeno) (MAXWELL; KRUGER; STANOSZ, 1997). Contrariamente, estresses abióticos podem conferir resistência a certos patógenos. Por exemplo, Plantas de tomate submetidas à seca tiveram redução de 50% na infecção pelo fungo *Botrytis cinerea* e a propagação do fungo *Oidium neolycopersici* foi suprimida (ACHUO; PRINSEN; HOFLE, 2006).

2 HIPÓTESE

O feijão-de-corda (*Vigna unguiculata)* genótipo CE-31 (ou Pitiúba), tolerante ao estresse hídrico e suscetível ao CPSMV, responde ativamente a esses estresses (individualmente ou simultaneamente impostos) por meio de reprogramação gênica, que leva a alterações no perfil proteômico. Entretanto, as respostas da planta aos estresses individuais são diferentes de quando os estresses são simultaneamente impostos e, esta segunda condição, aumenta a agressividade dos sintomas da doença.

3 OBJETIVOS

3.1 Objetivo geral

Estudar as respostas fisiológicas e bioquímicas/moleculares do feijão-de-corda (*Vigna unguiculata*, genótipo CE-31) quando individualmente ou simultaneamente submetido ao estresse hídrico e infecção pelo CPSMV.

3.2 Objetivos específicos

- Avaliar parâmetros fisiológicos referentes à fotossíntese nas folhas de feijão-de-corda, genótipo CE-31, submetidas individualmente ou simultaneamente ao estresse hídrico e infecção pelo CPSMV;

- Determinar a cinética enzimática de proteínas antioxidantes (superóxido dismutase, catalase, ascorbato peroxidase e guaiacol peroxidase) e PR-proteínas (glucanase e quitinase) em folhas de feijão-de-corda, genótipo CE-31, submetidas individualmente ou simultaneamente ao estresse hídrico e infecção pelo CPSMV;

- Verificar o acúmulo de peróxido de hidrogênio em folhas de feijão-de-corda, genótipo CE-31, submetidas individualmente ou simultaneamente ao estresse hídrico e infecção pelo CPSMV;

- Quantificar o CPSMV em folhas de feijão-de-corda, genótipo CE-31, submetidas individualmente ou simultaneamente ao estresse hídrico e infecção pelo CPSMV;

- Extrair proteínas de folhas de feijão-de-corda, genótipo CE-31, submetidas ao estresse hídrico e/ou infectadas com o CPSMV, e obter, por espectrometria de massas (LC-MS/MS), perfis com boa reprodutibilidade entre as repetições técnicas e biológicas;

- Analisar os perfis espectrométricos obtidos de proteínas de folhas de feijão-de-corda, genótipo CE-31, usando o programa *Data Analysis*;

- Identificar, classificar e categorizar as proteínas diferencialmente acumuladas em folhas de feijão-de-corda, genótipo CE-31, submetidas individualmente ou simultaneamente ao estresse hídrico e infecção pelo CPSMV.

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CAPÍTULO II

Drought increases cowpea (*Vigna unguiculata* [L.] Walp.) susceptibility to Cowpea severe mosaic virus (CPSMV) at early stage of infection

Rodolpho Glauber Guedes Silva

Drought increases cowpea (*Vigna unguiculata* [L.] Walp.) susceptibility to Cowpea severe mosaic virus (CPSMV) at early stage of infection

Rodolpho G. G. Silva, Ilka M. Vasconcelos, Thiago F. Martins, Anna L. N. Varela, Pedro F. N. Souza, Ana K. M. Lobo, Fredy D. A. Silva, Joaquim A. G. Silveira, Jose T. A. Oliveira*

Department of Biochemistry and Molecular Biology, Federal University of Ceara, CE, Brazil

* Corresponding author. Tel.: +55 85 33669823; fax: +55 85 33669789. E-mail address: jtaolive@ufc.br (J.T.A. Oliveira).

Abstract

This work aimed to examine the physiological and biochemical changes of a cowpea genotype (CE-31) drought-tolerant, but susceptible to Cowpea severe mosaic virus (CPSMV) in response to drought, CPSMV-infection, and to these combined stresses. Photosynthetic parameters related with gas exchange and fluorescence emission of photosystem II were measured. In addition, the activities of enzymes related to oxidative stress (superoxide dismutase, catalase, ascorbate peroxidase), and pathogenesis-related proteins (guaiacol peroxidase, β-1,3-glucanase, and chitinase) were measured at 2 and 6 days after CPSMV inoculation (DAI). Under the combined stresses, CPSMV did not affect the net photosynthesis, transpiration rate, and conductance. However, the internal CO_2 content was reduced and the water use efficiency increased at 2 DAI. Moreover, both the photochemical system and electron transport rate decreased at 2 DAI, although reversibly because there was no change in the potential quantum efficiency of photosystem II at 6 DAI. Regarding to the studied enzymes, the combined stresses did not induce alteration of SOD activity, diminished CAT at 2 DAI and 6 DAI, and transiently induced APX (2 DAI), as its activity diminished at 6 DAI, all in relation to control (carborundum injuried) plants, whereas the H_2O_2 content increased at 2 and 6 DAI. Overall there were changes in the activities of the studied PR-proteins in the experimental groups in relation to control. All together, it was concluded that the drought stress imposed increases the cowpea susceptibility to CPSMV at the early stage of infection (2DAI).

Keywords: Cowpea, *Vigna unguiculata*; CPSMV; drought; combined stresses

1 Introduction

Cowpea (*Vigna unguiculata*) is an important crop cultivated in Brazil especially in the North and Northeast regions. It represents a very important food source for people in developing countries of the tropics and subtropics due to its high protein (20 - 25%) and nutrient (carbohydrates, lipids, minerals and vitamins) contents, in addition to its palatability (Quass, 1995). In Asia, Africa and Latin America more than 10 million hectares are planted with cowpea (Nedumaran et al., 2013) with a global yield over 5 million tons per year, but still a low productivity of around 472.66 kg/ha (Sousa et al., 2015) due to abiotic and biotic stresses, which affect plant growth and consequently grain yield (Farooq et al., 2012; Kareem and Taiwo, 2007). Drought stress and virus infection, alone or in combination, in the cultivated fields are amongst the several biotic and abiotic stresses that affect cowpea.

Viruses are small obligate intracellular parasites that require the host cellular machinery to multiply, spread, and colonize the plant. The virus genome usually consists of a single or double stranded DNA or RNA, which is protected by protein shells known as capsids. The Cowpea Severe Mosaic Virus (CPSMV) is a member of the *Comovirus* genus, subfamily *Comovirinae*, family *Secoviridae* (International Committee on Taxomomy of Viruses, 2014 – ICTV, www.ictvonline.org/virustaxonomy.asp). CPSMV possesses a bipartite genome composed of two positive-sense single stranded RNA molecules, denominated RNA1 and RNA2 (formely RNA-B for bottom component, and RNA-M for middle component), both with a 5' linked protein VPg (Viral Protein linked to genome), and a 3' polyadenylate tail (Sijen et al., 1995). CPSMV causes a major and widespread viral disease that leads to huge losses in cowpea productivity worldwide (Kareem and Taiwo, 2007). The main symptoms of CPSMV infection are severe green yellow mosaic (chlorotic lesions) on leaves, leaf puckering and leaf area reduction (de Jager, 1979).

Water deficit is a major abiotic factor that causes negative impact on crop productivity (Farooq et al., 2012). However, some cowpea genotypes have moderate tolerance to drought, associated with stomatal control that minimizes water loss and functions as an adaptation mechanism (de Carvalho et al., 1998). Cowpea prevents tissue dehydration by maintaining high water potential

(ψL) and relative water content (RWC) when under water stress by means of stomatal closure (de Carvalho et al., 1998) leading to reduction in the CO_2 assimilation. Therefore, drought affects many physiological and biochemical processes promoting reductions of stomatal conductance, photosynthesis rate, cell division, and accumulation of reactive oxygen species (ROS).

ROS play important roles in the responses to biotic and abiotic stresses. Consequently, the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), amongst others, are fundamental to protect plant cells against the deleterious effects of excess ROS (Wu et al., 2006). Other crucial biochemical response to biotic and/or abiotic stresses is the increased synthesis of particular defense-related enzymes such as guaiacol peroxidases (POX), β-1,3-glucanases (GLU), and chitinases (CHI), which are grouped within the pathogenesis-related proteins (PR-Proteins) family (Nasser et al., 1990).

Under field conditions, besides to water deficiency, plants are often simultaneously attacked by pathogens and herbivores. The plant responses to each individual stress cannot be extrapolated when they occur in combination (Atkinson and Urwin, 2012), because abiotic stresses can either enhance or reduce tolerance to biotic stresses, and vice-versa. Mayek-Perez et al. (2002) observed increased transpiration rate and leaf temperature in *Phaseolus vulgaris* plants subjected to water stress and simultaneously infected with the fungus *Macrophomina phaseolina*, compared to plants only under water stress. On the other hand, drought stress acclimation in tomato increased the ABA content and enhanced resistance to the fungus *Botrytis cinerea* (Achuo et al., 2006).

In spite of being naturally exposed to several abiotic and biotic stresses in the field, to the best of our knowledge there is no report in the literature focusing on the cowpea responses to the combined drought and CPSMV-infection stresses. Therefore, in this present study, the physiological and biochemical responses of the cowpea genotype CE-31 submitted simultaneasly to both stresses were assessed in order to verify whether these stresses in combination alter drought tolerance and/or CPSMV susceptibility of this cowpea genotype used as a model. .

2 Material and methods

2.1 Plant material and growth conditions

Cowpea seeds (genotype CE-31, syn. Pitiuba), high susceptible to CPSMV, but drought tolerant, were supplied by The Brazilian Enterprise for Agricultural Research (EMBRAPA), Piaui, Brazil. Mature seeds were disinfected using 3% (v/v) sodium hypoclorite (0.05% active chloride) during 3 min, rinsed with distilled water and soaked in distilled water for 20 min. The selected seeds were germinated in Germtest® paper (28 x 38 cm) soaked with a volume of distilled water corresponding to twice its dry weight under sterile condition. The seeds were then allowed to germinate in a humid environment close to 100% relative humidity, in the dark, for two days. Next, the germinating seedlings were transplanted to 1.5 L plastic pots (3 seedlings per pots), containing vermiculite and river sand (1:9, m/m), this later previously washed exhaustively (7x) with tap water followed by distilled water (1x), and autoclaved (121 °C, 1.5×10^5 Pa, 30 min). The pots were kept under greenhouse conditions with the photosynthetic photon flux density (PPFD) varying from 300-650 µmoles m^{-2} s^{-1} (190SA quantum sensor, LI-COR, USA), a photoperiod of 12 h and temperatures of 27.0 ± 0.8 °C (night) and 31.0 ± 3.0 °C (day) and 79.8 ± 10.9% relative humidity. Plants were daily watered with distilled water until the sixth day and then watered with a nutrient solution described by Hoagland and Arnon (1950), modified by Silveira et al. (2001), diluted (1:10, v/v) with distilled water.

2.2 Drought stress and CPSMV inoculation

The drought stress imposed to cowpea (CE-31) plants was 25% of pot capacity. At 10 days after planting, irrigation was withdrawal and 4 days later 25% pot capacity was reached. Thereafter, until the end of the experimental period, irrigation was done with a given volume sufficient to keep the plants at this fixed pot capacity. The loss of water was evaluated by weighing the pots containing the substrate and plants. Plant groups that were not under water deficit remained at 70% pot capacity throughout the experiment.

The virus inoculums were prepared from the high susceptible cowpea genotype CE-31 plants infected with the isolate $CPSMV_{CE}$ (thereafter referred as CPSMV) obtained at Ceara State – Brazil. These infected plants were maintained in a greenhouse at the Laboratory of Plant Defense Proteins, Department of Biochemistry and Molecular Biology, Federal University of Ceara, Brazil, under the previously described conditions of temperature, light regime, light intensity, and humidity. Virus inoculums for mechanical transmission to receiving cowpea leaves were prepared by maceration of infected CE-31 cowpea leaves, showing typical severe mosaic symptoms, with 10 mM sodium phosphate buffer, pH 7.0, containing 0.01% (m/v) sodium sulfite, (1:10, m/v), and the resulting suspension mixed with 500-600 mesh carborundum powder used as abrasive. CPSMV inoculation was performed after 14 days of planting in fully expanded secondary leaves (first trifoliate leaves). Leaves were manually infected with the virus particles by rubbing the inoculum on the adaxial and abaxial leaf surfaces using the tips of the index finger and thumb with hands protected with surgical gloves. The control treatment consisted of inoculation of cowpea leaves with inoculums prepared from health (uninfected) CE-31 plants using the same above solution used to prepare the true inoculums.

Three experimental groups and a control were used in this study: (V) CPSMV inoculated cowpea (CE-31) plants; (D) cowpea plants (CE-31) subjected to drought; (DV) cowpea plants (CE-31) submitted to drought and infected with CPSMV; (C) control cowpea plants (CE-31) that were free from both stresses. The secondary leaves were collected 2 and 6 days after CPSMV inoculation. Drought stress and CPSMV inoculation periods are outlined in Fig. 1.

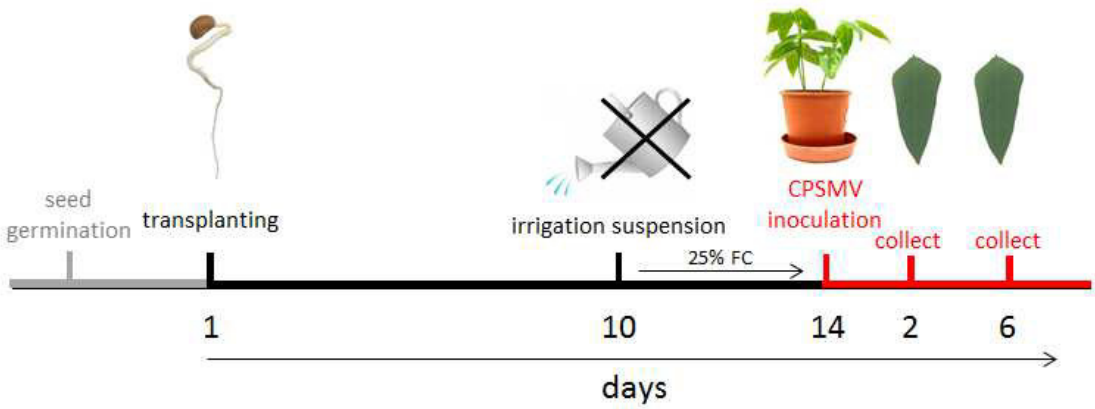

Fig. 1. Schematic diagram of drought stress imposition and CPSMV-inoculation of cowpea plants (CE-31 genotypes). PC: field capacity.

2.3 Leaf gas exchange and chlorophyll a fluorescence

Infrared gas analyzer (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA) equipped with a leaf chamber fluorometer (LI-6400-40, LI-COR, Lincoln, NE, USA).was used to measure gas exchange and chlorophyll *a* fluorescence. Leaf CO_2 assimilation rate (P_N), transpiration rate (E), stomatal conductance (g_S) and intercellular CO_2 partial pressure (C_i) were measured. The environmental conditions inside the IRGA chamber during the measurements were: PPFD of 1000 µmol m^{-2} s^{-1}, 28 ºC, air vapor pressure deficit of 1.0 ± 0.2 kPa, and air CO2 partial pressure of 38 Pa. The instantaneous carboxylation and water use efficiency were calculated as P_N/Ci and P_N/E, respectively. Fluorescence measurements were performed by the pulse method saturation (Schreiber et al. 1994) in leaves exposed to light, after they have being adapted to dark conditions for 30 min. The light saturation pulse intensity and duration were 8000 µmol m^{-2} s^{-1} and 0.7 s, respectively. The amount of blue light was adjusted to 10% of PPFD to maximize stomatal opening (Flexas et al., 2008). The parameters evaluated were: maximum PSII quantum yield [Fv/Fm = (Fm-Fo)/Fm]; effective PSII quantum yield [ΔF/Fm' = (Fm'-Fs)/Fm']; and flow of current photons directed to PSII (apparent electron transport rate) [ETR = (ΔF/Fm' x PPFD x 0.5 x 0.85)]. To evaluate the electron transport rate (ETR) at PSII level, 0.5 was used as the excitation energy of the fraction distributed to PSII, and 0.85 as the light fraction absorbed by leaves. Fm and Fo are the

maximum and minimum fluorescence, respectively, of dark-adapted leaves; Fm' and Fs are the maximum and steady-state fluorescence, respectively, in the light-adapted leaves (Schreiber et al., 1994).

2.4 Enzyme extraction and assays

The protein extract preparation from cowpea secondary leaves submitted to each treatment and time point were macerated under an ice bath with 0.05 M sodium acetate buffer (pH 5.2) containing 0.15 M NaCl, 0.002 M EDTA and 1% (m/v) PVPP (1:4, m/v) during 10 min. The homogenate was centrifuged (12000 x *g*, 4 °C, 20 min) and the supernatant collected and dialyzed exhaustively (two changes with ten times the supernatant volume at a 6-h interval) against the extraction buffer for 48 h at 4 °C. The dialyzed extract obtained was used for enzyme activity determination. The protein content was determined by the dye-binding method of Bradford (1976), using bovine serum albumin as the standard.

SOD (EC 1.15.1.1) activity was determined (Van Rossum et al., 1997) in 96-well plates measuring the ability of the protein extract to inhibit the NBT photoreduction. The reaction mixture consisted of 0.05 mL the enzyme extract, 0.03 mL distilled water, 0.01 mL 1 M potassium phosphate buffer, pH 7.8, 0.02 mL 1 mM EDTA, 0.02 mL 0.25% (v/v) Triton X-100,, 0.02 mL 0.13 M L-methionine, 0.02 mL 7.50 x 10^{-8} M riboflavin and 0.020 mL 10^{-3} M NBT. A first reading of microplate was performed before exposure of the reaction mixture to light. Subsequently, the reaction was initiated by exposure of the plates to a 32 W fluorescent lamp. Absorbances at 630 nm were read at 1 min intervals up to for 5 min in a microplate reader (Automated Microplate Reader ELX800, Bio-Texas Instruments® model, Inc.) The blank proof contained all reagents except the enzyme extract, which was replaced by ultrapure water, in order to promote maximum NBT reduction. One unit of SOD activity (AU) was defined as the amount of sample required to promote 50% inhibition of the NBT photoreduction rate. SOD activity was expressed in units per g fresh tissue (AU $g^{-1}T$).

CAT (EC. 1.11.1.6) activity was determined as previously described (Sudhakar et al., 2001). The enzyme extract (0.2 mL) was incubated at 30 °C during 10 min with 2800 mL of 0.02 M H_2O_2 prepared in 0.05 M potassium-

phosphate buffer (pH 7.0). The decrease in absorbance at 240 nm was measured at 20-second intervals up to 2 min and CAT activity expressed in units per gram fresh tissue (AU g^{-1}T). Decrease of 1.0 absorbance unit per min was assumed to be 1.0 unit of POX activity (UA), and was expressed as the change in absorbance per min per g of plant fresh tissue (AU g^{-1} T).

APX (EC 1.11.1.11) activity was measured (Koshiba, 1993) adding 0.1 mL of the enzyme extract to 0.8 mL of 0.05 M potassium phosphate buffer, pH 6.0, containing 5×10^{-4} M ascorbic acid and preincubating this mixture at 30 °C. The reaction was initiated by adding 0.1 mL of a 2×10^{-3} M hydrogen peroxide (H_2O_2) solution. The decrease in the absorbance measured at 290 nm for 2 min at 20 s intervals was measured as ascorbate oxidation index. APX activity was expressed in units per gram fresh tissue (AU g^{-1}T). Decrease of 1.0 absorbance unit per min was assumed to be 1.0 unit of APX activity (UA), and was expressed as the change in absorbance per min per g of plant fresh tissue (AU g^{-1} T).

GLU (EC 3.2.2.6) activity was determined (Boller, 1992) using laminarin as the substrate. The laminarin solution (2×10^{-3} g L^{-1}) was dissolved in ultrapure water, heated to 60 °C for 10 min, and dialyzed exhaustively against ultrapure water to remove free glucose. Aliquots of 0.05 mL of the enzyme extract were incubated with 0.05 mL of 50 mM sodium acetate buffer, pH 5.2, and 0.9 mL laminarin solution at 50 °C for 30 min. After addition of the appropriate reagents to the reaction mixture product, according to Boller (1992), absorbance readings were taken at 520 nm and the amount of glucose monomers released by the enzyme action was determined using a standard curve constructed from known concentrations of D-glucose, ranging from 3 to 30×10^{-6} g mL^{-1}. β-1,3-glucanase activity was expressed in nanokatal per g fresh tissue (nkat g^{-1} T). 1.0 nkat was equivalent to 1.0 nmol of glucose released per second, under the test conditions.

CHI (EC 3.2.1.14) activity was determined (Boller, 1992; Reissig et al., 1955) by measuring the N-acetyl-D-glucosamine (NAG) monomers released by the hydrolytic action of the enzyme on colloidal chitin used as substrate. To determine total chitinolytic activity, 0.250 mL of the enzyme extract, previously diluted (5-fold) with sodium acetate buffer (50 mM, pH 5.2) was incubated with 0.250 mL of colloidal chitin (10 g L^{-1}) at 37 °C under constant stirring for 1 h.

Next the reaction mixture was heated at 98 °C in a water bath, for 5 min, and immediately cooled in an ice bath. For blank samples the substrate (colloidal chitin) was added after the heat denaturation of the enzyme (98 °C, 5 min). The tubes were centrifuged at 10000 x *g* for 10 min at 5 °C, and 0.3 mL aliquots of the supernatant were carefully removed and transferred to new microtubes to which 0.01 mL β-glucuronidase enzyme (Sigma) solution previously diluted (10x) with ultrapure water. Samples were further incubated at 37 °C for 1 h under constant agitation, heated at 98 °C in a water bath for 5 min, and cooled in an ice bath. To 0.31 mL of the hydrolysates 0.19 mL of 50 mM sodium acetate buffer, pH 5.2, and 0.1 mL of 0.6 M potassium tetraborate were added. The reaction mixture was heated to 98 °C, for 5 min, and cooled before addition of 1.0 mL 4-(dimethylamino)benzaldehyde (DMAB, Sigma) prepared by dissolving 10.0 g DMAB in 100 mL glacial acetic acid containing 12.5% of 11.5 M hydrochloric acid. This solution was diluted twice with acetic acid at the time of use. This mixture was incubated at 37 °C for 20 min, and absorbance readings immediately taken at 585 nm. Increasing NAG concentrations (0.1 – 0.6 M) were used to obtain a standard curve required to calculate the amount of NAG released in the reaction. The chitinase activity was expressed in nanokatal per g fresh tissue (nkat g^{-1} T). 1.0 nkat was equivalent to 1.0 nmol of released NAG per second, under the test conditions.

POX (EC 1.11.1.7) activity was performed using guaiacol and H_2O_2 as substrates according to Urbanek et al. (1991). The reaction mixture consisted of 0.980 mL aliquots of 0.05 M sodium acetate buffer, pH 5.2, 0.5 mL 0.02 M guaiacol, and 0.5 mL 0.06 M H_2O_2, incubated at 30 °C. Aliquots of 0.02 mL of each enzyme extract were added to this above mixtture and the absorbance was immediately read at 480 nm (spectrophotometer Thermo Scientific, Genesys 10S UV-VIS). The increase in absorbance due to formation of the compound 3,3'- dimethoxy-4,4'-bifenolquinona was monitored for 2 min in 20 s intervals. The variation of 1.0 absorbance unit per min was assumed to be 1.0 unit of POX activity (UA), and was expressed as the change in absorbance per min per g of plant fresh tissue (AU g^{-1} T).

2.5 Hydrogen peroxide

For qualitative determination of hydrogen peroxide (H_2O_2), 3'-3'-diaminobenzidine (DAB) staining was performed in cowpea leaves (Thordal-Christensen et al., 1997). The DAB solution was prepared in ultrapure water, the pH adjusted to 3 with HCl, and heated at 50 °C for 1 h. After cooling, pH was adjusted to pH 4 using NaOH. The cowpea shoot was cut at 2 cm above the cotyledon insertion region with a stiletto, and the cut end immersed in a solution containing 1 mg DAB mL^{-1}. After 8 h of incubation, cowpea leaves were rinsed exhaustively with distilled water, and depigmented by incubation in trichloroacetic acid solution (1.5 g L^{-1}) prepared in a mixture of ethanol and chloroform (3:1, v/v) for 24 h. H_2O_2 leaf accumulation was visualized under an optic microscope (Olympus BX 60 Microscope System).

The leaf H_2O_2 content was quantified according to the colorimetric method (Gay et al., 1999). Secondary cowpea leaves were powdered in liquid N_2 and the material was homogenized in 0.05 M borax-borate buffer, pH 8.4 (1:4, m/v), for 3 min. Subsequently, the homogenate was centrifuged (12000 x *g*, 4 °C, 20 min) and the supernatant obtained used in the assay. The reaction mixture consisted of 0.02 mL of the supernatant and 1.0 mL of xylenol orange solution [100 µL solution "A" (0.025 M ferrous sulfate, 0.025 M ammonium sulphate, and 2.5 M sulfuric acid) + 10 mL of solution "B" (1.25 x 10^{-4} M xylenol orange + 10^{-4} M sorbitol)] prepared at the time of assay. This mixture was homogenized and incubated for 30 min at room temperature (23 ±2 °C), and the absorbance was measured at 560 nm. A standard curve with known concentrations of hydrogen peroxide (0–10.0 x 10^{-3} mmol H_2O_2/1.2 mL) was performed to determine the H_2O_2 amount in the cowpea leaves.

2.6 Molecular detection and quantification of CPSMV

2.6.1 RNA isolation and reverse transcription

RNA isolation of secondary cowpea leaves was carried out using the NucleoSpin® RNA Plant kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The RNA yield was determined by absorbance ratio taken at 260/280 nm, using a Nanodrop spectrophotometer. The isolated RNA was stored at -80 ℃ until use. The RNA obtained was transcribed into

cDNA using ImProm-II™ reverse transcriptase (Promega). The RNA sample was mixed with oligo(dT)s diluted in RNase-free water. The mixture was incubated at 70 °C for 5 min, and immediately chilled at 4 °C for 5 min under an ice bath. The Reverse Transcription Mix was added and annealing was performed at 25 °C for 5 min, followed by extension at 42 °C for 60 min. Reverse transcriptase reaction was stopped by holding the mixture at 70 °C for 15 min.

2.6.2 Product amplification and quantification

Degenerated primers corresponding to a conserved genome sequence specific for the coat protein gene of the genus *Comovirus* were used: 5'-YTCRAAWCCVYTRTTKGGMCCACA-3'- R and 5' -GCATGGTCCACWCAGGT-3' – F (Brioso et al., 1996). Different amounts of cDNA (1.5 x 10^{-9}, 3 x 10^{-9}, and 6 x 10^{-9} g) were added to 9 µL PCR reaction mix containing the primers (1 µL 0.020 M reverse and 1 µL 0.020 M forward), 0.1 µL 0.010 M dNTPs and 0.1 µL (1 unit μL^{-1}) GoTaq® DNA Polymerase (Promega), 2 µL 5x PCR-buffer, and 4.8 µL RNase-free water. The reaction conditions were an initial denaturation step for 3 min at 94 °C followed by 27 cycles of denaturation for 1 min at 94 °C, primer annealing for 30 s at 41 °C, and elongation for 45 s at 72 °C. The final elongation step was done for 5 min at 72 °C. The PCR-products were loaded onto a 1.2% (m/v) agarose gel, dyed with ethidium bromide and analyzed, after electrophoresis, under UV-light. Different amounts of cDNA were eletrophoresed to establish the saturation concentration of the band in the agarose gel in order to allow quantitative comparison between the band intensities corresponding the virus coat protein gene present in the leaves of the virus-infected and (V) and, simultaneously, drought exposed and virus-infected plants (DV). The band intensities were evaluated using the Gel Analyzer software, and the area values of the peaks compared.

2.7 Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) and the means compared using the Tukey's test at a confidence level of 5% ($p \leq 0.05$).

3 Results

3.1 Plant growth and CPSMV symptoms

Cowpea plants subjected to drought stress (experimental group D) reduced their size (Fig. 2A) and leaf area (Fig. 2B) compared to well watered plants (control group). The symptoms of CPSMV infection were not visible 2 days after virus inoculation (2 DAI). However, 6 days after CPSMV inoculation (6 DAI) the characteristic mosaic symptoms were visible at naked eyes (Fig. 2B).

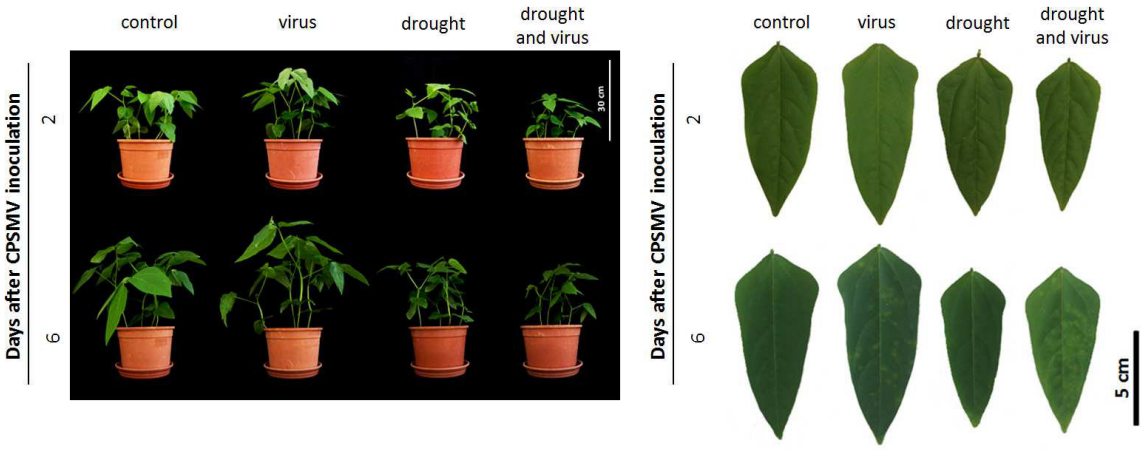

Fig. 2. Representative images of cowpea plants (A) and leaves (B) subjected to water stress (25% of pot capacity) or/and CPSMV infection at 2 and 6 days after CPSMV inoculation (DAI).

3.2 Leaf gas exchange and chlorophyll a fluorescence

The drought stress imposed to cowpea plants (experimental group D) caused prominent decreases in P_N, E, g_S, and Ci (Fig. 3A, B, C, and D, respectively) compared to controls in both times analyzed. However, the decreases were more pronounced in g_S and Ci for plants treated with both stresses (experimental group DV) at 6 DAI. Drought unstressed plants only

inoculated with CPSMV (experimental group V) showed a significant increase of P_N, but E decreased at 2 DAI compared to controls (Fig. 3A).

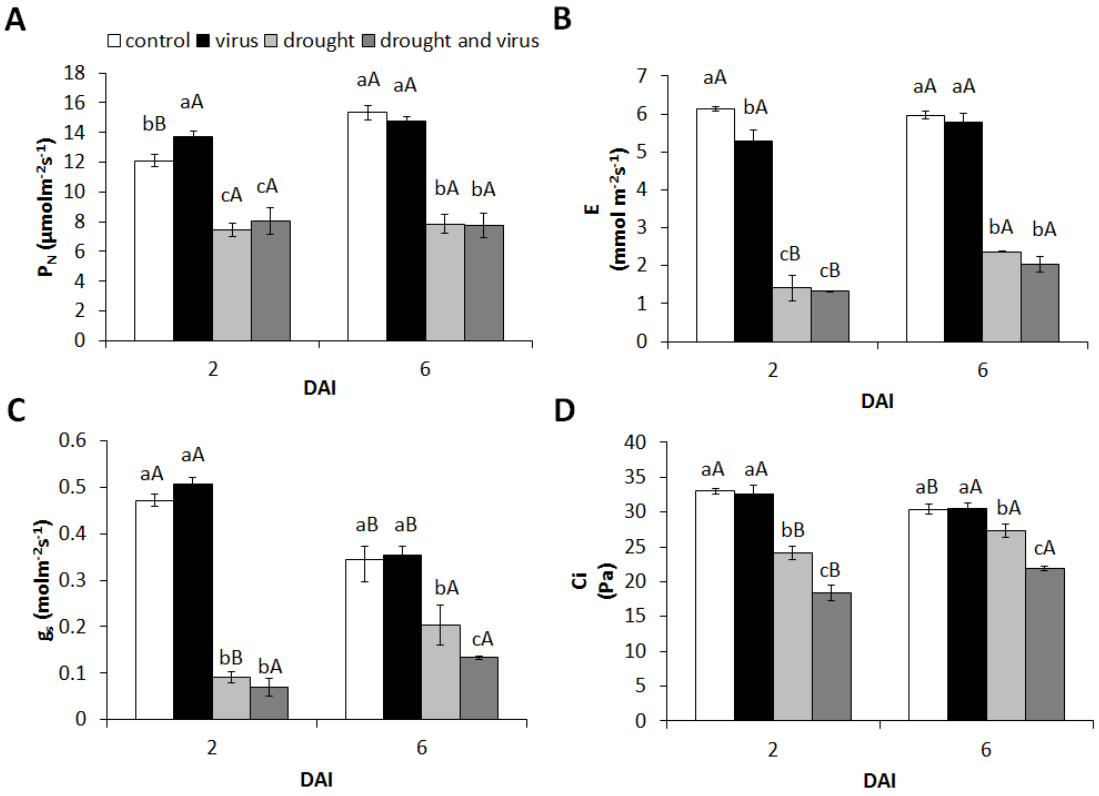

Fig. 3. (A) Net photosynthesis, (B) transpiration rate, (C) stomatal conductance, and (D) internal CO_2 partial pressure in cowpea leaves (CE-31genotype) subjected to water stress (25% of pot capacity) or/and CPSMV infection at 2 and 6 days after CPSMV inoculation (DAI). Each data point represents the mean of three independent replicates ± standard error (bar). Different letters denote significant differences ($p \leq 0.05$) among means. Small letters represent comparisons among treatments and capital letters represent comparison over time.

Instantaneous carboxylation efficiency (P_N/Ci) (Fig. 4A) and water use efficiency (P_N/E) (Fig. 4B) increased significantly ($p \leq 0.05$) in V, D and DV compared to control plants at 2 DAI. At 6 DAI instantaneous carboxylation efficiency significantly ($p \leq 0.05$) decreased in D and DV compared to control

(Fig. 4A) and water use efficiency increased significantly (p≤.05) in both cowpea groups (D and DV) exposed to drought stress (Fig. 4B).

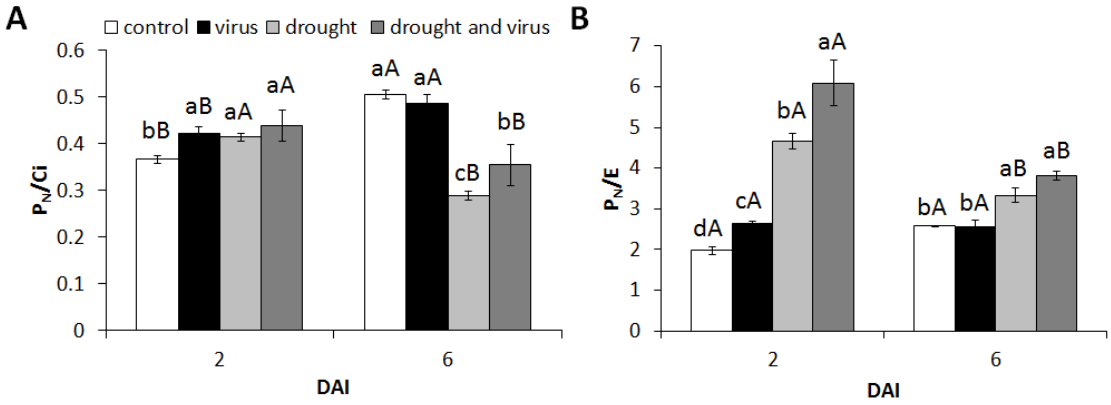

Fig. 4. (A) Instantaneous carboxylation efficiency and (B) water use efficiency (P_N/E) in cowpea leaves (CE-31 genotype) subjected to water stress (25% of pot capacity) or/and CPSMV infection at 2 and 6 days after CPSMV inoculation (DAI). Each data point represents the mean of three independent replicates ± standard error (bar). Different letters denote significant differences ($p \leq 0.05$) among means. Small letters represent comparisons among treatments and capital letters represent comparison over time.

The effective quantum efficiency of photosystem II (ΔF/Fm`) was not different among the experimental and control groups at 2 DAI (Fig. 5A), but increased significantly ($p \leq 0.05$) in control and in the V group at 6 DAI (Fig. 5A) to values significantly higher than those for D and DV groups, being D higher than DV at this time point. In relation to ETR, the data for the groups V, D, and DV were similar, but lower compared to control, at 2 DAI (Fig. 5B). At 6 DAI, there was a significant decrease of ETR values for D and DV compared with C and V. Moreover, compared to the respective figures at 2 DAI, ETR values were higher for D, V, and control group, but not for DV which was similar. Regarding to the potential quantum efficiency of photosystem II (Fv/Fm) (Fig. 5C) no differences were observed at 2 and 6 DAI among the studied plant groups.

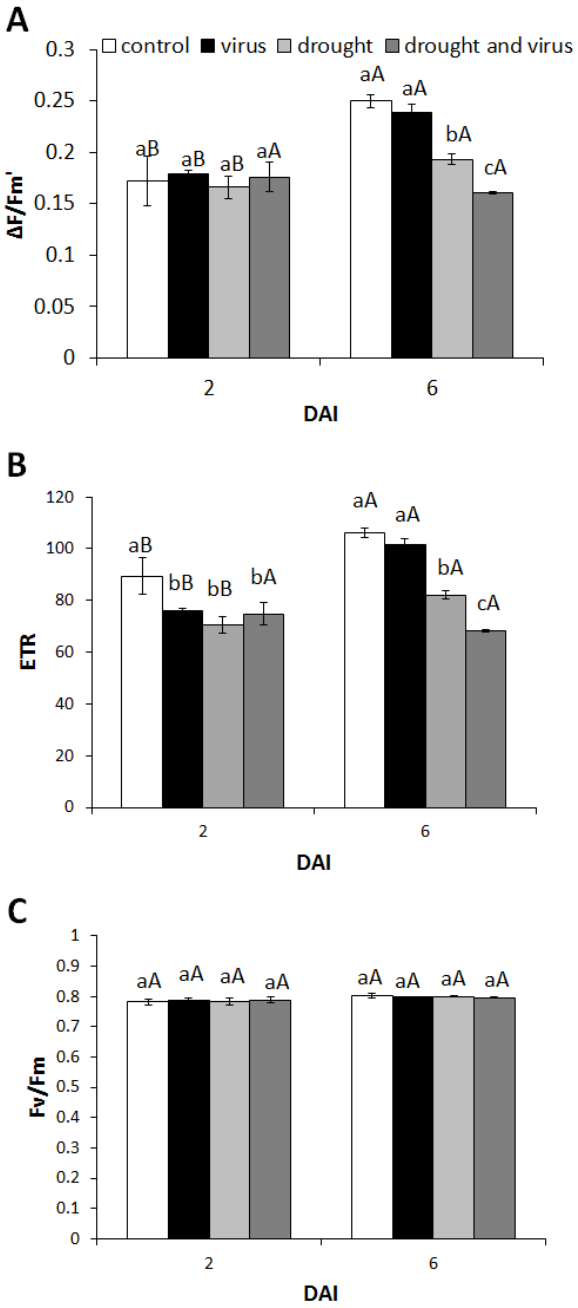

Fig. 5. Effective quantum efficiency of photosystem II (A), electron transport rate (B) and potential quantum efficiency of photosystem II (C) in cowpea leaves (CE-31 genotype) subjected to water stress (25% of pot capacity) or/and CPSMV infection at 2 and 6 days after inoculation (DAI). Each data point represents the mean of three independent replicates ± standard error (bar). Different letters denote significant differences ($p \leq 0.05$) among means. Small letters represent comparisons among treatments and capital letters represent comparison over time.

3.3 Enzymes

SOD activity of V plants was significantly ($p \leq 0.05$) lower (6%) than the other groups (C, D, and DV) at 2 DAI. However, at 6 DAI all values were similar within the plant groups and compared to the values at 2 DAI (Fig. 6A).

CAT activity decreased significantly ($p \leq 0.05$) in V (65.2%), D (30.0%) and DV (43.4%) at 2 DAI compared to control (Fig. 6B). Similarly, at 6 DAI, CAT activity was 39.6%, 70.5%, and 93.2% lower in V, D and DV compared to control, respectively (Fig. 6B). Comparing the two time points the control group values of CAT were unchanged, but whereas V was higher at 6 DAI, D and DV showed lower values.

APX activity increased significantly ($p \leq 0.05$) in D (19.4%) and elevated very prominently in DV (191.1%) at 2 DAI compared to control (Fig. 6C). By contrast, at 6 DAI the APX values were around 44%, 34%, and 30% significantly ($p \leq 0.05$) lower in V, D and DV compared to control, respectively (Fig. 6C). Moreover, the APX values measured at 6 DAI were significantly ($p \leq 0.05$) lower than the corresponding values at 2 DAI.

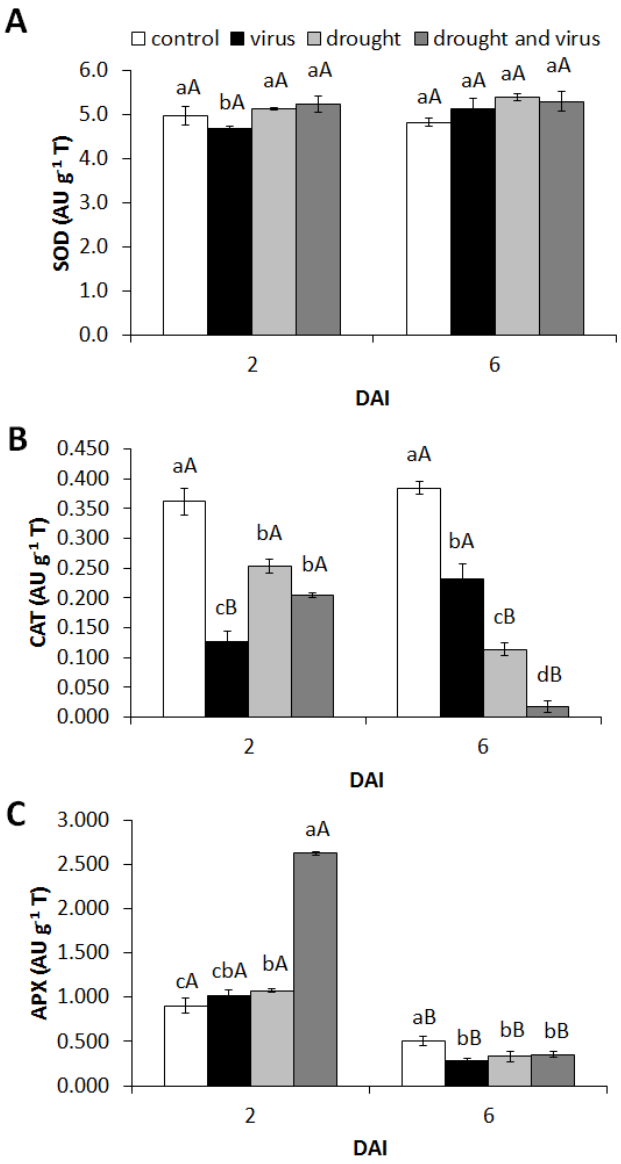

Fig. 6. Activity of SOD (A), CAT (B), and APX (C) in cowpea leaves (CE-31 genotype) subjected to water stress (25% of pot capacity) or/and CPSMV infection at 2 and 6 days after inoculation (DAI). Each data point represents the mean of three independent replicates ± standard error (bar). Different letters denote significant differences ($p \leq 0.05$) among means. Small letters represent comparisons among treatments and capital letters represent comparison over time.

GLU activity increased significantly ($p \leq 0.05$) only in D (75.4%) at 2 DAI compared with control, which had similar values compared with V and DV. At 6 DAI, the GLU activity of V was still higher (50.82%) compared with the respective control as it was D (19.67%) at this time point (Fig. 7A).

CHI activity increased significantly ($p \leq 0.05$) in V (≈1000%) and even more for DV (≈1500%) compared to the control at 2 DAI (Fig. 7B). At 6 DAI, CHI activity of DV remained still higher (250%) compared to control plants (Fig. 7B), but very much lower than the value at 2 DAI.

Elevated POX activity ($p \leq 0.05$) of 86.5% and 111.2% was observed for V and D plant groups, respectively, but not for DV, as compared with control, at 2 DAI, (Fig. 7C). At 6 DAI, the POX activity values of V, D and DV were 82.3%, 64.7%, and 75.3% higher, respectively, compared ($p \leq 0.05$) to control (Fig. 7C). Comparing the two time points studied, the POX activity of control plants remaining unaltered as did the V group, but D was slight lower and DV much higher at 6 DAI compared with 2 DAI.

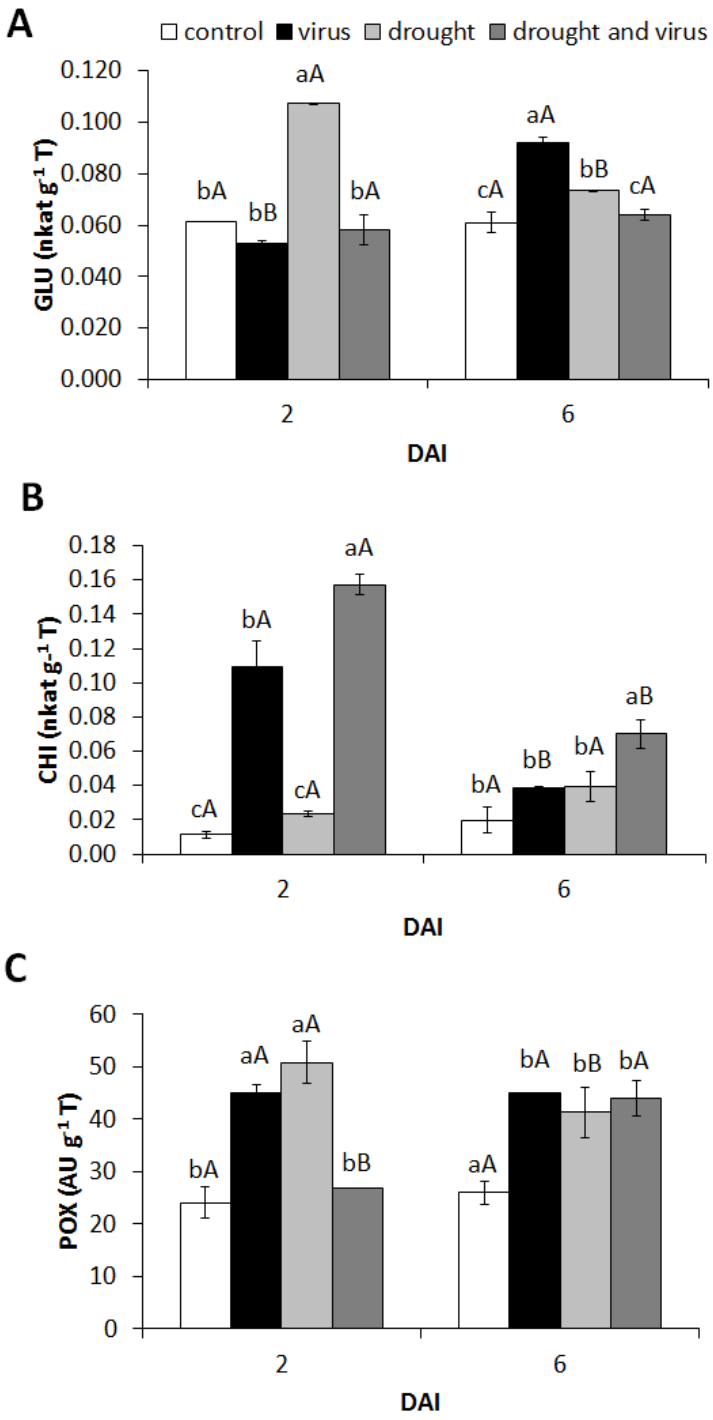

Fig. 7. Activity of GLU (A), CHI (B), and POX (C) in cowpea leaves (CE-31 genotypes) subjected to water stress (25% of pot capacity) or/and CPSMV infection at 2 and 6 after post inoculation (DAI). Each data point represents the mean of three independent experiments ± standard error (bar). Different letters denote significant differences ($p \leq 0.05$) among means. Small letters represent comparisons among treatments and capital letters represent comparison over time.

3.4 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) accumulation was observed by optical microscopy visualization for D and DV cowpea leaves, but not for V, at 6 DAI compared to control (Fig. 8A). Quantitatively (Figure 8B), there was significant ($p \leq 0.05$) increases in the H_2O_2 contents in V, D, and DV groups at 2 (31.6%, 42.35%, 30.47%, respectively) and 6 days (83.72%, 161.97%, 286.95%, respectively), as compared with the respective controls. However, this increase in H_2O_2 content was very much prominent for DV at 6 DAI.

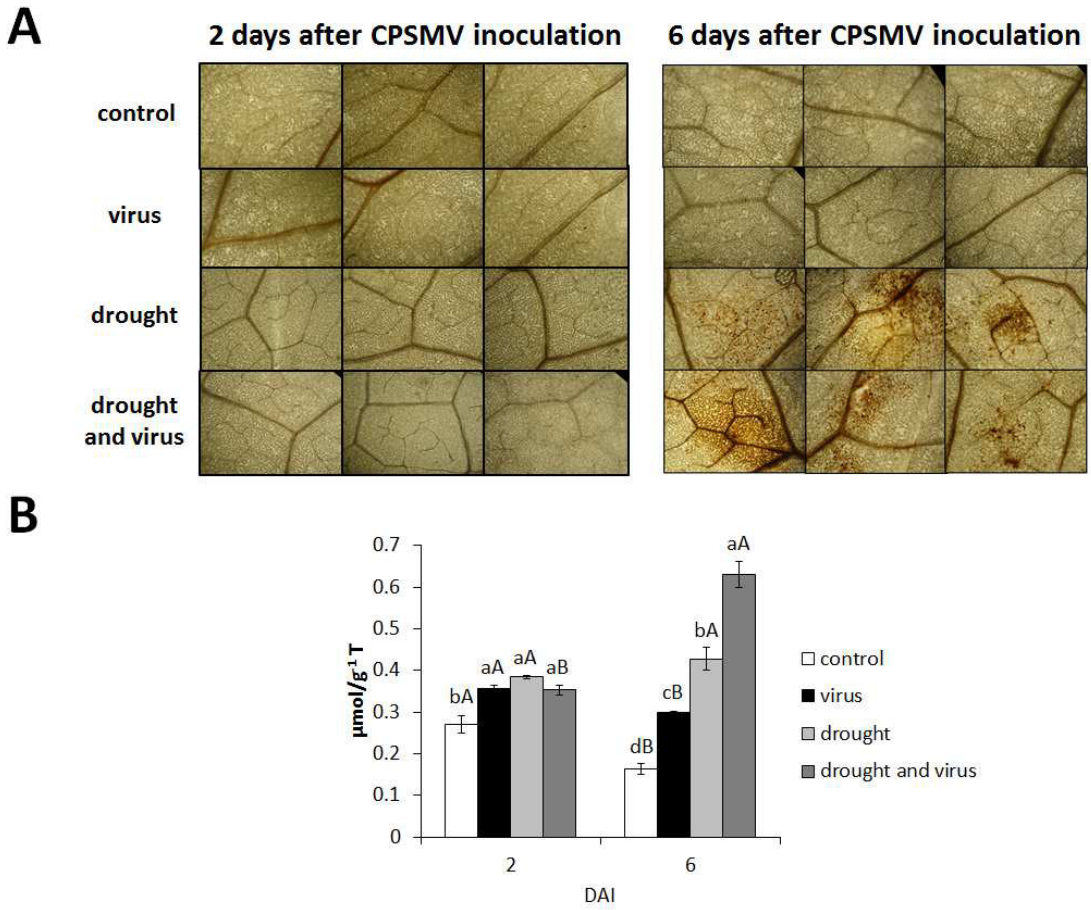

Fig. 8. H_2O_2 detection by optical microscopy after DAB staining (A) and quantification (B) in cowpea leaves (CE-31 genotype) subjected to water stress (25% of pot capacity) or/and CPSMV infection at 2 and 6 days after inoculation (DAI). Each data point represents the mean of three independent replicates ± standard error (bar). In (B) different letters denote significant differences ($p \leq 0.05$) among means. Small letters represent comparisons among treatments and capital letters represent comparison over time.

3.5 Molecular detection of CPSMV

The amount of cDNA used to quantify the intensity of the band in the agarose gel was 1.5 ng. Control and plants subjected to drought did not show any amplification (Fig. 9A). However, there was a significant ($p \leq 0.05$) increase (≈28%) in the raw peak area of DV compared to V at 2 DAI (Fig. 9B). At 6 DAI the virus particle number were similar amongst V and DV, but higher compared to those at 2 DAI.

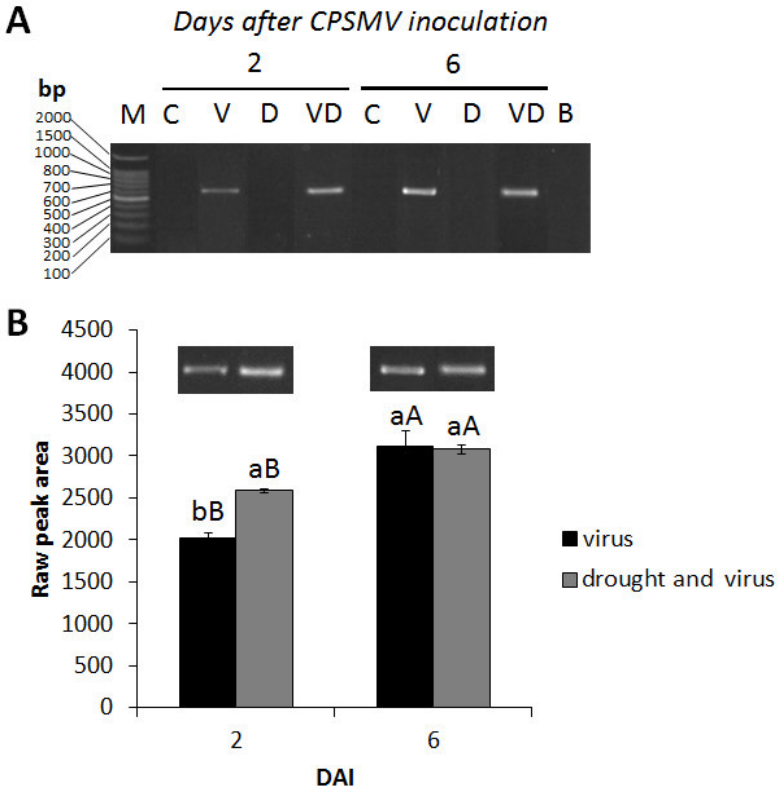

Fig. 9. Quantification of CPSMV by band intensity. (A) Agarose gel electrophoresis of the PCR product amplified using 1.5 ng cDNA from cowpea leaves infected with CPSMV (2 and 6 days after inoculation) and subjected to drought. (B) Raw peak area data from the agarose gel obtained with the help of the Gel Analizer software. Each data point represents the mean of three independent experiments ± standard error (bar). In (B) different letters denote significant differences ($p \leq 0.05$) among means. Small letters represent comparisons among treatments and capital letters represent comparison over time.

4 Discussion

Drought stress reduced cowpea size (CE-31 genotype) and leaf area (Fig. 2). It is known that drought stress substantially interferes with plant growth and development affecting the crop yield by alteration of various physiological and biochemical processes, such as photosynthesis, respiration, and hormonal

balance (Farooq et al., 2012). By contrast, CPSMV infection did not affect the plant size and leaf area up to 6 DAI (Fig. 2).

Our study revealed that P_N, E, g_S, and Ci decreased in the cowpea plants subjected to water deficit (Fig. 3). However, these parameters were stabilized from 6 days of drought, confirming the tolerance to water deficiency of CE-31 genotype. Sengupta et al. (2013) also observed decline in these parameters in *Vigna radiate* under progressive drought stress. Stomatal closure is the main factor limiting photosynthesis in plants subjected to water stress (Cornic, 2000) and it is considered as a mechanism of tolerance to avoid water losses (Schachtman and Goodger, 2008). In contrast, CPSMV infection of CE-31 did not alter these above physiological parameters (Fig. 3).

The observed reductions in the gas exchange parameters of D and DV plant groups at 6 DAI suggested that photosynthesis was inhibited by stomatal and biochemical limitation. However the biochemical limitation was more important as P_N/Ci decreased in D and DV (Fig. 3 and 4). In our study, the water use efficiency (P_N/E) increased significantly ($p \leq 0.05$) in D and DV plants (Fig. 4B), which reinforces that the CE-31 genotype is drought tolerant even when infected with CPSMV (Fig. 4).This result is consistent with the study conducted by Sengupta et al. (2013), in which there was increase in P_N/E in *Vigna radiata* subjected to water stress at 3 and 6 days after the water withdrawal.

At 6 DAI, the combined stresses (drought + CPSMV infection) imposed to CE-31 compromised the photochemical system (Fig. 5A) and the electron transport rate of the DV plant group (Fig. 5B). However, these negative effects were apparently reversible because no change in Fv/Fm was noticed, indicating that the photosynthetic electron transport system remained intact (Fig 5C).

One of the earliest cellular events associated with plant defense against biotrophic organisms, following successful pathogen recognition, is the oxidative burst, in which excess ROS (superoxide anion, hydrogen peroxide, hydroxyl radical, and others) are generated, leading to hypersensitive response (HR) and programmed cell death (PCD), which restrict pathogen spreading, particularly in the case of biotrophics as viruses, from the infection site to other plant tissues (Torres et al., 2006). ROS can also restrict the spread of pathogens by both directly killing and strengthening the plant cell wall via cross-linking of

glycoproteins (Moshe et al., 2012; Torres et al., 2006). Increased hydrogen peroxide (H_2O_2) content observed for DV compared to V at 6 DAI (Fig. 8) might have resulted from the attempt of cowpea (CE-31 genotype) to restrict CPSMV particles to spread throughout the whole plant and cause a systemic infection. However, such increase in H_2O_2 content observed in V and DV cowpea plants at 2 and 6 DAI was not sufficient to prevent viral infection (Fig. 8). On the other hand, excess H_2O_2 is cytotoxic and can provoke host cellular death, particularly through lipid peroxidation and membrane damage, and also interfere negatively with the photosystem II reaction center. For instance, our current study showed lower effective quantum efficiency of photosystem II in DV plant group at 6 DAI (Fig. 5A).

The *de novo* biosynthesis of antioxidant enzymes such as SOD, CAT, and APX is an important biochemical event to avoid the deleterious effects of ROS in plant cells (Wu et al., 2006). SOD in plants acts as an antioxidant by catalyzing the dismutation (disproportionation) of two superoxide anions into molecular oxygen and H_2O_2. H_2O_2, besides playing crucial roles in plant defense by acting as a direct toxic substance and restrict pathogen invasion by strengthening the plant cell wall, is involved in various signal transduction which lead to expression of several resistance genes. Our present study showed decreased SOD activity in V compared to DV and control plants at 2 DAI, but similar activity to other groups at 6 DAI (Fig. 6A). Wu et al. (2013) also showed low SOD representation in a susceptible cultivar of maize infected with sugarcane mosaic virus (SCMV) using a proteomic approach. Likewise, Hernández et al. (2001) found decreased SOD activity in a susceptible apricot infected with plum pox virus (PPV). Regarding to DV cowpea plants, SOD activity was similar to that of D and control at 2 DAI, suggesting that the drought stress alone imposed to cowpea overcome the CPSMV-induced slightly lower SOD levels in V plants (2 DAI), which might have a consequence in the H_2O_2 generation in those plants to which the combined stresses were imposed. Indeed, D and DV plant groups had much higher H_2O_2 generation than V plants.

CAT catalyzes the decomposition of H_2O_2 to water and oxygen. CAT activity of cowpea leaves decreased significantly in V, D, and DV plant groups compared to control, both at 2 and 6 DAI (Fig 6B). These findings might be associated with the very higher H_2O_2 contents of V, D, and DV compared to

control, particularly at 6 DAI (Fig. 8). Decrease in CAT activity in response to water stress was also reported in sunflower and wheat (Zhang and Kirkham, 1994). However, it is more common to see increases in CAT activity in response to drought (Hu et al., 2013).

The ascorbate-glutathione cycle, in which ascorbate peroxidase (APX) plays a key role, is the major H_2O_2 detoxifying system in plant cells (Giacomelli et al., 2007). Compared with CAT, which has low affinity for H_2O_2 (mM range), APX has higher affinity (µM range) and thus it is able to scavenge low amounts of H_2O_2 and fine regulates its levels in cells (Mittler, 2002). In cowpea leaves, the APX activity increased significantly in DV at 2 DAI compared to control (Fig. 6C). Perhaps this cowpea plant group attempted to keep ROS levels under tight control by regulating the increased levels of H_2O_2 (Fig. 8). However, the APX activity reduced at 6 DAI (Fig. 6C), when a prominent increase in H_2O_2 levels was observed in DV compared to the other treatments (Fig 8). This effect of drought on cowpea APX was previously found for other plants (Bacelar et al., 2007). Clarke et al. (2002) reported increased virus replication and development in the compatible interaction of *Phaseolus vulgaris* and white clover mosaic virus as the antioxidant enzyme activities decreased, highlighting the important role of these enzymes in response to viral infections.

Several studies have shown that virus spread in plants is also dependent on the balance between callose synthesis and hydrolysis. Callose accumulation slows the virus movement through the plant tissues (Radford and White, 2001). Thus, decrease in GLU activity, which degrade callose, causes delay in the spread of plant viruses cell-to-cell. In the present study, increased GLU activity in V plants compared to control at 6 DAI may facilitate cell-to-cell movement of CPSMV. Contrarily, GLU decreased activity in DV cowpea plants compared to V at 6 DAI (Fig. 7A) might have resulted in the accumulation of callose, slowing the cell-to-cell spreading of CPSMV.

Increased CHI activity for V and DV plants compared to control was observed at 2 DAI (Fig. 7B) with DV even higher than V. At 6 DAI, the CHI activity decreased considerably compared to 2 DAI, but in DV it remained higher than for the other plant groups. The first report on CHI induction by virus infection was provided independently by Van Loon and Van Kammen (1970) and Gianinazzi et al. (1970) in *Nicotiana tabacum* leaves infected with tobacco

mosaic virus (TMV). Elvira et al. (2008) identified four different chitinases up-represented in *Capsicum chinense* L3 plants infected with pepper mild mottle virus (PMMoV) by proteomic study. Glu and CHI are two PR-proteins that often work together in plants against pathogen attacks. However no function was yet attributed to chitinases in response to viral infection.

It is well know that upon biotic and abiotic stresses plant cell wall reinforcement by lignification is one of the defense responses (Barros *et al*., 2015). Peroxidases are enzymes directly involved with the lignification process and thus strengthening of the plant cell wall. In addition to participate in the lignifications process of cell walls, POX also scavenges H_2O_2, contributing to the ROS homeostase. In the present study, drought stress and viral infection alone, but not in combination, induced POX activity at 2 DAI (Fig. 7C). However, at 6 DAI, POX activity was increased in all experimental plant groups (V, D, and DV). Increased POX activity in response to drought may result in changes in the mechanical properties of the cell wall, which may be attributed to adaptive mechanisms to drought stress (Lee et al., 2007). Regarding to viral infection, Dória *et al*. (2015) observed high POX activity in sweet orange (Westin variety) infected with *Citrus tristeza virus* (CTV).

In this current study, quantification of the amplified product corresponding to CPSMV coat protein showed increased accumulation for DV compared to V at 2 DAI (Fig. 9). This indicates that drought stress facilitated the viral infection in cowpea. Combined stresses in plants can cause positive or negative effects depending on the kind and intensity of the stress imposed and the pathogen. For example, *Phaseolus vulgaris* subjected to drought and infected with *Macrophomina phaseolina* increased transpiration rate and leaf temperature compared to plants subjected only to water stress (Mayek-Perez et al., 2002). Likewise, hybrid poplar (*Populus nigra* x *P. maximowiczii*) subjected to drought and infected with *Septoria musiva* developed larger cankers (Maxwell et al., 1997). By contrast, Achuo et al. (2006) demonstrated that imposition of water stress to tomato increased the ABA content and enhanced resistance to the fungus *B. cinerea*.

In conclusion, this study suggests that the drought stress increases the cowpea susceptibility to CPSMV infection at 2 DAI. However, the amount of virus particles in the cowpea tissues of DV, as seen by RT-PCR, was similar to

V at 6 DAI, suggesting that the cowpea plants submitted to the combined stresses tried to overcome the drought-induced higher susceptibility of the DV group. Contrarily, CPSMV infection seems to have not increased the negative effects of drought on the tolerant CE-31 cowpea genotype. These results confirm that responses to the combined stresses could not be taken additively, indicating that such responses cannot be extrapolated from the study of individual stresses.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was financially supported by the Council for Advanced Professional Training (CAPES), the National Council for Scientific and Technological Development (CNPq, grant 308107/2013-6), the Scientific and Technological Development Support Program (PADCT), and the Research Council of the State of Ceara (FUNCAP, grant 2155/PRONEX). Silva, R. G. G. acknowledges a Doctoral grant from CAPES.

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CAPÍTULO III

Label-free quantitative proteomics reveals differentially regulated proteins in the cowpea leaves under biotic and abiotic stresses

Rodolpho Glauber Guedes Silva

Label-free quantitative proteomics reveals differentially regulated proteins in the cowpea leaves under biotic and abiotic stresses

Silva, R. G. G.[a], Vasconcelos, I. M. [a], Varela, A. L. N.[a], Silva, F. D. A. [a], Oliveira, K. S.[b], Franco, O. L.[b], Silveira, J. A. G.[a], Oliveira, J. T. A.[a]*

[a] Biochemistry and Molecular Biology Department, Federal University of Ceara, CE, Brazil.

[b] Centro de Análises Proteômicas e Bioquímicas, Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, Brazil.

* Corresponding author. Biochemistry and Molecular Biology Department, Federal University of Ceara, CE, Brazil, Laboratory of Plant Defense Proteins, Av. Mister Hull, P.O. Box: 60451 Fortaleza, CE, Brazil. Tel.: +55 85 33669823; fax: +55 85 33669789. E-mail address: jtaolive@ufc.br (J.T.A. Oliveira).

Abstract

In this work, we study the proteomic changes in cowpea (*Vigna unguiculata*, cv. CE-31) secondary leaves of plants submitted to drought, challenged with CPSMV, and to these combined drought stress and virus-infection by label free LC-ESI-MS/MS. Out of 571 identified proteins, 117 were differentially accumulated. These proteins were categorized into different groups according to physiological functions: energy and metabolism, photosynthesis, protein folding and metabolism, cell redox homeostasis, regulation factors and RNA processing, response to stress, lipid biosynthesis, structural protein, pyridoxine biosynthetic process and plant defense. In the group inoculated with CPSMV alone, down-representation of proteins involved with energy/metabolism and photosynthesis at 6 days after CPSMV inoculation (DAI) appears to be important for the cowpea susceptibility to the viral disease. Moreover, up-representation of translation elongation factor 1A (eEF-1A) plays a role in viral replication. Down-representation of proteins involved with photosynthesis in cowpea plants under drought is consistent with the limitation of photosynthesis in plants exposed to this kind of stress. The combination of drought and CPSMV infection recruited various proteins responsive to stresses such as heat shock 70 kD protein, 17.7 kDa class I small heat shock protein and glycine-rich RNA-binding protein, 6 DA. All together, the findings provided by this current study have great relevance for the understanding of the molecular mechanisms involved in the response of a CPSMV susceptible cowpea genotype (CE-31) infected with this virus and subjected to drought, alone or in combination.

Keywords: *Vigna unguiculata*, cowpea, drought, virus, combined stresses, proteomics

1 Introduction

Plants are often exposed in nature to various abiotic (extreme temperatures, high salt, drought) and biotic stresses (pathogen infection and herbivory). Two or more of these combined abiotic and biotic stresses influence each other depending on certain factors such as the stress nature, exposure time, and stress severity (Atkinson and Urwin, 2012). Nevertheless alone or in combination, the stresses generated losses in crops worldwide (Suzuki *et al.*, 2014).

After recognizing that are under stress (biotic and/or abiotic), plants trigger defense mechanisms that, in general, include activation of specific ion channels at the cell membrane, kinase cascades, reactive oxygen species (ROS) generation, phytohormone accumulation, and genetic reprogramming to adequate the plant metabolism to launch the counterattack in order to minimize the damage caused by stresses (Fraire-Velázquez *et al.*, 2011; Laloi *et al.*, 2004; Spoel and Dong, 2008; Fujita *et al.*, 2006).

Cowpea (*Vigna unguiculata* [L.] Walp) is a legume (*Fabaceae*) with high nutritional value grown mainly in Asia, Africa and Latin America, where they are grown more than 10 million hectares (Nedumaran *et al.*, 2013) with a production of more than 4.5 million tons per year (Singh *et al.*, 2003). This productivity could be higher if this important crop was free from biotic and abiotic stresses as drought and viral infection, for example.

Drought is one of the major abiotic stresses that lead to retardation of plant growth and development and low crop yield (Farooq *et al.*, 2012), which could reach over 50% in many grain crops (Boyer, 1982; Bray *et al.*, 2000; Parry, 2012). However, it is known that cowpea has some tolerance to water stress (Dadson *et al.*, 2005). Some of the biochemical and physiological responses of cowpea to cope with drought include stomatal closure, decreased CO_2 assimilation rates, and accumulation of soluble sugars (Souza *et al.*, 2004).

Within the viruses that infect cowpea, *Cowpea severe mosaic virus* (CPSMV) is considered one of the most important (Booker *et al.*, 2005). CPSMV is an isometric virus belonging to the genus *Comovirus.* Its genome has two molecules of positive-sense single stranded RNA (RNA 1 and RNA 2). These RNA molecules possess a protein known as virus genome-linked protein

(VPg) at the 5' end and a poly (A) tail at the 3' end (Chen and Bruening, 1992). RNA 1 molecule encodes proteins required for viral replication, while RNA 2 encodes two capsid proteins and a protein involved in cell-to-cell movement (Chen and Bruening, 1992).

Under field conditions, CPSMV transmission occurs through vectors, including *Ceratomia arcuata*. The main symptoms of CPSMV infection are chlorotic lesions that characterize the formation of the mosaic, malformation and leaf puckering and leaf size reduction (de Jager, 1979).

In this work, label free proteomic was employed to study the protein profile changes in cowpea leaves (*Vigna unguiculata*, cv. CE-31) challenged concomitantly by CPSMV and/or drought stress 2 and 6 days post virus inoculation.

2 Material and methods

2.1 Plant material and growth conditions

The Brazilian Agricultural Research Corporation (EMBRAPA), Piaui, Brazil, provided the cowpea seeds of the CE-31 genotype (syn. Pitiuba), which is highly susceptible to CPSMV, but drought tolerant. Mature seeds were disinfected using 3% sodium hypochlorite (0.05% active chloride) during 3 min, rinsed with distilled water, and soaked in distilled water for 20 min. The selected seeds were germinated under sterile condition in Germtest® paper (28 x 38 cm) previously moistened with a volume of distilled water corresponding to twice its dry weight. The seeds were germinated in chambers maintained near 100% relative humidity (RH) at 23 ± 2 °C, in the dark, for three days. Germinating seeds were transferred to 1.5 L plastic pots (3 seedlings per pots), containing vermiculite + river sand (1:9, m/m). The river sand was previously washed exhaustively (7x) with tap water, before being autoclaved (121 °C, 9.8 x 10^4 Pa, 30 min) with distilled water (1x). The pots were kept under greenhouse conditions with the photosynthetic photon flux density (PPFD) varying from 300-600 µmoles m^{-2} s^{-1}, 12 h photoperiod, temperatures of 27.0 ± 0.8 °C (night) and 31.0 ± 3.0 °C (day), and 79.8 ± 10.9% RH. Irrigation was done with distilled water up to the sixth day after transplanting to the pots and next with the

nutrient solution described by Hoagland and Arnon (1950), modified by Silveira *et al*. (2001), 1:10 (v/v) diluted with distilled water, until the end of the experimental period.

2.2 CPSMV inoculation

The inoculum for the mechanical transmission of the virus was prepared from the extract of infected cowpea leaves (cv. CE-31). Cowpea leaves presenting the mosaic symptoms were macerated using a graal and pistil with 0.01 M phosphate buffer, pH 7.0, containing 0.1% (m/v) sodium sulfite (1:10, leaf mass/v). To this suspension the abrasive carborundum (600 mesh) was added at 1:10 (m/v) ratio and this virus suspension used as inoculum. CPSMV inoculation was performed after 14 days of planting in fully expanded secondary cowpea leaves (first trifoliate leaves) from CE-31 plants used in the experiments. Inoculation was manually done with hands protected by surgical gloves. The virus suspension was collected and rubbed against the adaxial and abaxial leaf surfaces with the aid of the thumb and indicator finger. The control treatment consisted of rubbing the leaves with the carborundum containing phosphate buffer used above, but virus-free.

2.3 Drought treatment

The time required for the substrate (vermiculite + river sand) contained in the pots, where cowpea plants were sowing, to reach 25% of pot capacity (stress used in this study) was four days. Thus, for submitting plants to drought stress at this pot capacity, pots that had been daily watered were deprived of the nutritive solution four days before the CPSMV inoculation. The loss of water on the substrate was evaluated by weighing the pots containing the substrate and plants. The groups that were not under water deficit remained with 70% pot capacity throughout the experiment.

Three experimental groups and a control were used in this study: plants inoculated with CPSMV; plants subjected to drought; plants submitted to drought and simultaneously infected with CPSMV; and plants that were free from both stresses (control). Secondary leaves were collected 2 and 6 days

after CPSMV inoculation (DAI). Three biological replicates were performed for each treatment.

2.4 Proteomic analysis

2.4.1 Preparation of protein samples

Protein extraction was performed according to the methodology described by Yao *et al.*, (2006) with modifications. 2 g of cowpea secondary leaves collected as previously described were pulverized in liquid nitrogen with the aid of a graal and pistil and 15 ml of 10% (v/v) trichloroacetic acid (TCA) containing 2% (v/v) β-mercaptoethanol in pure acetone was added for homogenization. After centrifugation at 15000 x *g*, 15 min, 4 °C, the supernatant was discarded and the precipitate washed three times with 2% (v/v) β-mercaptoethanol in acetone (ice cold) and centrifuged (15000 x *g*, 15 min, 4 °C). The precipitate obtained was solubilized with 6 ml 100 mM Tris-HCl, pH 8, containing 30% (m/v) sucrose, 2% (w/v) sodium dodecyl sulfate (SDS), 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM polyvinyl polypyrrolidone (PVPP) and centrifuged at 10000 x *g* at 4 °C for 10 min. To the resulting supernatant an equal volume of Tris-phenol (phenol solution equilibrated with 10 mM Tris HCl, pH 8.0, with 1 mM EDTA, Sigma) was added and the mixture centrifuged (10000 x *g*, 4 °C, 10 min). The upper phenol phase was removed and 6 volumes of solution of 100 mM ammonium acetate in methanol were added and gently mixed. After incubation for 2 h at -20 °C, the mixture was centrifuged (15000 x *g*, 15 min, 4 °C) and the precipitate obtained washed three times with 80% (v/v) cold acetone and dried under silica gel in a dessicator. The precipitate was resuspended in 7 M urea and 2 M thiourea in 50 mM ammonium bicarbonate and the protein content of samples determined using the Quant-iT™ Protein Assay Kit, and the QubitTM fluorometer (Invitrogen Molecular Probes, USA).

2.4.2 Preparation of peptide samples

To every protein sample obtained as above, 20 µL of 50 mM ammonium bicarbonate and 50 µL of RapiGest SF (Waters) solution were added, the mixture shaken, incubated at 80 °C for 15 min, and centrifuged for 15 s at 10000 x *g*. Subsequently, 5 µL 100 mM dithiothreitol were added to the sample, the mixture vortex agitated and incubated at 60 °C for 30 min. After the incubation time, the samples were allowed to reach the room temperature (23 ± 2 °C) and centrifuged at 10000 x *g*, 15 s, 25 °C. Next, 5 µL 300 mM iodoacetamide were added to the samples, which were vortex agitated again and incubated for 30 min at room temperature (23 ± 2 °C), in the dark. After this time period, 20 µL of a trypsin (Sequencing Grade Modified Trypsin, Promega) solution (10^{-3} mg mL^{-1} concentration) prepared in 50 mM ammonium bicarbonate were added, mixed, and the samples incubated at 37 °C for 15 h to allow protein digestion. After hydrolysis, the samples were centrifuged at 21000 x *g*, 6 °C, for 30 min, the resulting supernatants containing the tryptic peptides transferred to microcentrifuge tubes and water evaporated in a vacuum concentrator at 30 °C (Eppendorf™ Vacufuge™ Concentrator). Finally, the tryptic peptides were resuspended in 50% (v/v) acetonitrile containing 0.1% (v/v) formic acid, centrifuged for 5 min at 21000 x *g*, 25 °C, and subsequently submitted for mass spectrometry analysis.

2.4.3 Mass Spectrometry

LC-ESI-MS/MS experiments were performed on HPLC (fast type) Shimadzu (Kyoto, Japan) coupled to a mass spectrometer type micrOTOF ESI-Q III (Bruker Daltonics, Bremen, Germany). Separation of peptides was performed in an XR-ODS C18 column (2.0 mm x 30 mm x 2.2 µm; Kyoto, Japan). Mobile phase solvents were 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The gradient elution used was 5% B for 5 min; 5%-50% B for 50 min; 50%- 95% B for 10 min; 95% B for 8 min; 95-5% B for 1 min, and 5% B for 7 min, at 400 $\mu L.min^{-1}$ flow rate. Analyses were performed with the mass spectrometer operating in the positive ESI linear mode with, the following parameters: ESI voltage of 4.5 kV; nitrogen (N_2) used as a nebulizer gas (4.0 Bar); dry gas flow of 8 L min^{-1} at a dry temperature of 200 °C.

The precursor ion scan range was 300-3000 m/z followed by five MS/MS scans. Three independent runs were performed for each sample.

2.4.4 Data analysis and database searching

The mass fragmentation spectra (MS/MS) data files generated were analyzed using the Data Analysis software version 4.0 (Bruker Daltonics, Germany). The spectra were deconvoluted by the deconvolute tool to determine peptides charge. The data were imported into the Biotools software (BrukerDaltonics, Germany) where the searches were conducted using the MASCOT algorithm (Matrix Science, London, United Kingdom, version 2.3). Searches were performed using *Glycine max* (taxid: 3847), *Phaseolus vulgaris* (taxid: 3885), and *Vigna unguiculata* (taxid: 3917) protein databases obtained from NCBI. Moreover, the VigGS database (Sakai *et al*., 2016) was searched (March 15, 2016) to validate protein identification using the above database. Searching parameters were: trypsin selected as the proteolyitc enzyme; carbamidomethylation of cysteine as a fixed modification; oxidized methionine as variable modifications. Precursor ion mass tolerance (MS) and fragment ion mass (MS/MS) tolerance were ± 0.2 Da. Proteins were considered identified, when the peptide score was greater than the minimum score for 95% confidence ($p < 0.05$), which was obtained by the match of each peptide generated against the theoretical fragmentation profile (MASCOT). Every ion had to be present in all three replicates as inclusion criteria.

2.4.5 Protein quantification

Proteins present in every experimental group were quantified to compare relative abundances using the Data Analysis Tool version 4.0 (Bruker Daltonics, Germany). Quantification was based on the ion peak area relative to the triplicate experiments carried out. For each identified protein, three ions were selected by the Extracted Ion Chromatogram (EIC) tool. The area of each ion was calculated by the Integrate Chromatogram (IC) tool, and the values obtained used to compare between the plant groups (control vs. stress). Only exclusive proteins from control, virus, drought, and combined stresses, and

overlapping proteins that showed at least 2-fold up-accumulation (≥ 2.0) and decrease (≤ 0.6) in concentration in relation to control were considered differentially represented.

2.4.6 Gene ontology and subcellular location

Gene ontology (GO) annotation of the identified proteins was conducted using a free access database (http://www.uniprot.org/). Plant-mPLoc version 2.0 software (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) was employed to predict subcellular location.

2.4.7 Interaction network of differentially accumulated proteins

The protein–protein interaction network was established online using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) v. 10.0 program (http://string-db.org/). Differentially up- and down-represented cowpea proteins were blasted against *Solanum lycopersicum* that includes direct (physical) and indirect (functional) relationships supported by associations derived from the following sources: genomic context, high-throughput experiments, co-expression, and previous knowledge from bibliographical resources.

3 Results

Label-free LC-MS/MS analysis was used to characterize the differential abundance of cowpea leaf proteins after plants (CE-31 cowpea genotype) were subjected to CPSMV infection, drought, and the simultaneously combined stresses (drought and CPSMV-infection). In these analyses, 571 proteins were identified in cowpea leaves across the studied treatments, using the NCBI database. In total, 117 proteins were differentially accumulated; 50 up- and 67 down-represented (Table 1). The relationships among the experimental groups (virus, drought, and combined drought stress and virus-infection) compared to control are represented in Fig. 1.

The differentially accumulated proteins common to the three treatments at 2 DAI (virus, drought, and combined drought stress and virus-infection) were ATP synthase CF1 alpha subunit and peroxisomal catalase (Table 1 and Fig. 1). At 6 DAI, the differentially accumulated proteins common to the three treatments were fructose-1,6-bisphosphatase, photosystem I reaction center subunit III, photosystem II CP47 chlorophyll apoprotein, photosystem II protein D2, photosystem II protein D1, translation elongation factor-TU, CSP41a, digalactosyldiacylglycerol synthase 1, and putative pyridoxine biosynthetic enzyme (Table 1 and Fig. 1).

Identified proteins were categorized into nine major groups based on their putative biological functions (Fig. 2): energy and metabolism; photosynthesis; cell redox homeostasis; protein folding and metabolism; regulation factors and RNA processing; response to stress; lipid biosynthesis; pyridoxine biosynthetic process; structural protein; and plant defense. Subcellular localization prediction indicates that these proteins located in chloroplasts, cytoplasm, cell membrane, nucleus, peroxisome, endoplasmatic reticulum, Golgi apparatus and mitochondrion (Fig. 3). Determining subcellular localization is important for understanding protein function and is a critical step in genome annotation

Table 1 – Identity of differentially accumulated proteins from the secondary leaves of cowpea plants exposed to drought (D), infected with CPSMV (V), and submitted to the combined stresses (DV)

Protein name	NCBI accession no.	Reference organismo	Subcellular localization	Responsive to	Protein fold change (stress vs. control)*	
					At 2 days	At 6 days
Energy & metabolism						
ATP synthase CF1 alpha subunit	gi|349589861	*Vigna unguiculata*	Chloroplast	Virus Drought drought and virus	4.14 4.38 5.76	
glyceraldehyde-3-phosphate dehydrogenase	gi|543176550	*Phaseolus vulgaris*	Cell membrane Chloroplast	Drought	+	
vacuolar H+-ATPase subunit A, partial	gi|66816974	*Vigna unguiculata*	Cell membrane Mitochondrion.	Drought drought and virus	+	+ +
PREDICTED: probable fructose-bisphosphate aldolase 2, chloroplastic-like	gi|356538694	*Glycine max*	Chloroplast	Virus Drought drought and virus	 + +	- 3.56

PREDICTED: probable fructose-bisphosphate aldolase 2, chloroplastic-like	gi|356508188	*Glycine max*	Chloroplast	Virus		+
				Drought	-	
				drought and virus	-	
triosephosphate isomerase	gi|48773765	*Glycine max*	Chloroplast	Virus		+
				drought and virus	+	+
glyceraldehyde-3-dehydrogenase C subunit	gi|74475508	*Glycine max*	Cytoplasm	Virus		-
				Drought		-
				drought and virus	-	
PREDICTED: ATP synthase gamma chain, chloroplastic-like isoform 3	gi|356548957	*Glycine max*	Chloroplast	Virus		-
				drought and virus	-	

chloroplast ATP synthase gamma subunit, partial	gi|83744158	*Vigna unguiculata*	Chloroplast	Virus	-
ATP synthase CF1 epsilon subunit	gi|349589845	*Vigna unguiculata*	Chloroplast	Virus	-
fructose-1,6-bisphosphatase	gi|515747	*Glycine max*	Chloroplast	Virus	-
				Drought	-
				drought and virus	-
malate dehydrogenase, partial	gi|3192929	*Glycine max*	Chloroplast Mitochondrion.	Virus	-
				Drought	-
PREDICTED: glyceraldehyde-3-phosphate dehydrogenase, cytosolic	gi|356508778	*Glycine max*	Cytoplasm	Virus	+
fructose-bisphosphate aldolase	gi|40457267	*Glycine max*	Cytoplasm	Virus	+

glyceraldehyde-3-phosphate dehydrogenase	gi|543176466	*Phaseolus vulgaris*	Cytoplasm	Drought		-
fructose-bisphosphate aldolase 1	gi|543176716	*Phaseolus vulgaris*	Chloroplast	drought and virus		-
PREDICTED: phosphoribulokinase, chloroplastic-like	gi|356531479	*Glycine max*	Chloroplast	drought and virus		+
Photosynthesis						
photosystem I reaction center subunit II chloroplastic-like protein	gi|543177449	*Phaseolus vulgaris*	Chloroplast	Virus	-	
LHCII type I chlorophyll a/b-binding protein	gi|543176952	*Phaseolus vulgaris*	Chloroplast	drought and virus	0.39	
photosystem I subunit VII	gi|349589891	*Vigna unguiculata*	Chloroplast	Virus	-	

photosystem I reaction center subunit III	gi|543177275	*Phaseolus vulgaris*	Chloroplast	Virus	+	-
				Drought		-
				drought and virus	+	-
photosystem II CP47 chlorophyll apoprotein	gi|349589876	*Vigna unguiculata*	Chloroplast	Virus	+	-
				Drought		-
				drought and virus		-
photosystem II CP43 chlorophyll apoprotein	gi|349589903	*Vigna unguiculata*	Chloroplast	Virus		-
				Drought	-	-
				drought and virus		-
chloroplast oxygen-evolving enhancer protein	gi|558695637	*Phaseolus vulgaris*	Chloroplast	Drought	+	2.74
				drought and virus	+	

photosystem II D1 protein (chloroplast)	gi|984735	*Vigna unguiculata*	Chloroplast	Drought	+	-
				drought and virus		-
photosystem II protein D2	gi|349589854	*Vigna unguiculata*	Chloroplast	Virus		-
				Drought		-
				drought and virus	+	-
photosystem II protein D1 (chloroplast)	gi|112030956	*Phaseolus vulgaris*	Chloroplast	Virus		-
				Drought		-
				drought and virus		-
oxygen-evolving enhancer protein 1	gi|543176923	*Phaseolus vulgaris*	Chloroplast	Virus		+
				drought and virus		+

oxygen-evolving enhancer protein 2 chloroplastic-like protein	gi|543176871	*Phaseolus vulgaris*	Chloroplast	Virus	0.54	
Rubpcase	gi|18742	*Glycine max*	Chloroplast	drought and virus	0.04	0.30
Cell redox homeostasis						
peroxisomal catalase	gi|543176548	*Phaseolus vulgaris*	Peroxisome.	Virus Drought drought and virus	- - -	
peroxisomal glycolate oxidase	gi|543176674	*Phaseolus vulgaris*	Peroxisome.	Virus	-	
Peroxiredoxin	gi|11558242	*Phaseolus vulgaris*	Chloroplast Cytoplasm	Virus drought and virus	+ +	+

				Drought		-
Catalase	gi|18560	*Glycine max*	Peroxisome	drought and virus	+	
copper/zinc superoxide dismutase	gi|558695578	*Phaseolus vulgaris*	Chloroplast	drought and virus		+
Protein Folding & Metabolism						
glutamine synthetase	gi|452916816	*Vigna unguiculata*	Chloroplast Cytoplasm	Virus	+	
				drought and virus		+
glutamine synthetase precursor	gi|13877511	*Glycine max*	Chloroplast Mitochondrion.	drought and virus	-	
peptidyl-prolyl cis-trans isomerase	gi|543177006	*Phaseolus vulgaris*	Chloroplast Endoplasmic reticulum Extracell. Golgi apparatus Mitochondrion.	Virus		-

Cyclophilin	gi|829119	*Phaseolus vulgaris*	Cytoplasm	Virus		+
				drought and virus		+
Regulation factors or RNA processing						
EF-Tu (eEF1A)	gi|18776	*Glycine max*	Chloroplast	Virus	-	
				Drought	-	
				drought and virus		+
translation elongation factor-TU, partial	gi|2546954	*Glycine max*	Chloroplast	Virus	+	-
				Drought	+	-
				drought and virus		-
PREDICTED: chloroplast stem-loop binding protein of 41 kDa a, chloroplastic-like (CSP41a)	gi|356535978	*Glycine max*	Chloroplast Cytoplasm	Virus		-
				Drought		-
				drought and virus		-
eEF-1a	gi|18765	*Glycine max*	Cytoplasm Nucleus	Virus		+
elongation factor 1 beta (eEF-1B, formerly EF-Ts)	gi|308191651	*Vigna unguiculata*	Mitochondrion. Nucleus	drought and virus		+

Response to stress						
PREDICTED: stromal 70 kDa heat shock-related protein, chloroplastic-like (HSP70)	gi|356559803	*Glycine max*	Chloroplast	Virus	+	
				Drought		-
				drought and virus	+	
Heat Shock 70kD protein (HSP70)	gi|18663	*Glycine max*	Nucleus	Virus		+
				drought and virus		+
PREDICTED: glycine-rich RNA-binding protein 7-like	gi|356508388	*Glycine max*	Nucleus	drought and virus		+
glycine-rich RNA-binding protein	gi|5726567	*Glycine max*	Nucleus	drought and virus		+
17.7 kDa class I small heat shock protein (smHSP)	gi|154293473	*Vigna unguiculata*	Nucleus	drought and virus		+
heat shock protein 90-2 (HSP90)	gi|208964722	*Glycine max*	Cytoplasm	drought and virus		+

Structural protein						
actin-3-like protein	gi|543176885	*Phaseolus vulgaris*	Chloroplast Mitochondrion.	Virus		-
				Drought	+	-
				drought and virus	+	
Lipid biosynthesis						
digalactosyldiacylglycerol synthase 1	gi|76800640	*Vigna unguiculata*	Chloroplast Mitochondrion	Virus		-
				Drought		-
				drought and virus		-
Pyridoxine biosynthetic process						
putative pyridoxine biosynthetic enzyme	gi|10719739	*Phaseolus vulgaris*	Cytoplasm	Virus		-
				Drought		-
				drought and virus		-
Plant defense						
cowpea pathogenesis-related protein 3 (CpPR3)	gi|4850337	*Vigna unguiculata*		drought and virus	+	

* Signals “+” and “–” indicate that the protein was present or absent, respectively, at 2 and 6 DAI, as compared to the respective control group.

The empty spaces in the columns indicate that the protein has not been identified or changed in response to the corresponding treatment.

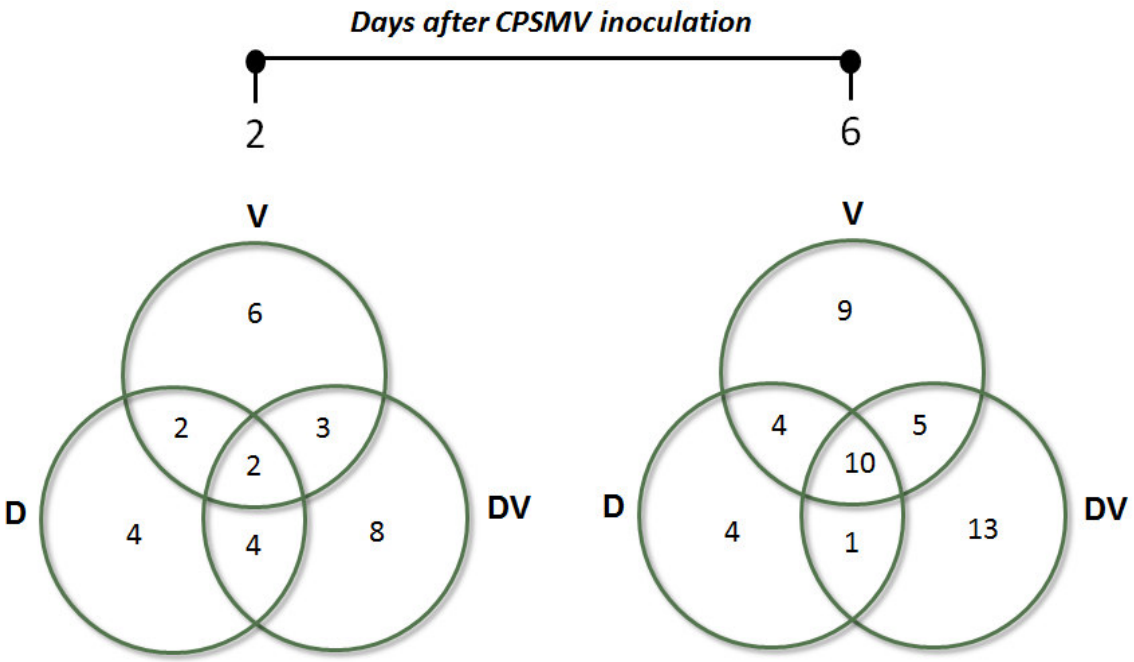

Fig. 1. Venn diagrams showing the number of differentially accumulated proteins from the secondary leaves of cowpea plants exposed to drought (D), infected with CPSMV (V), and submitted to the combined stresses (DV).

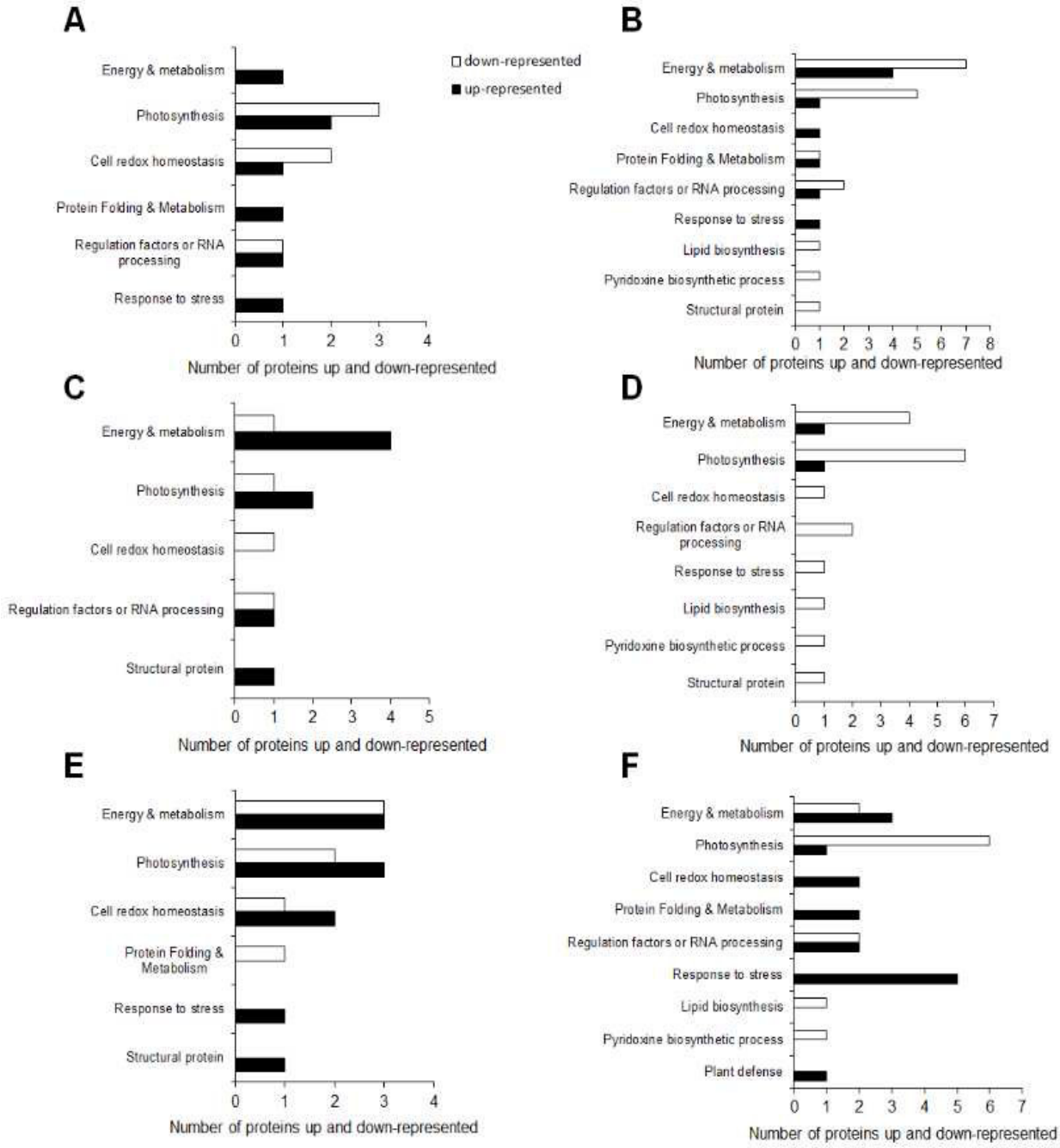

Fig. 2. Distribution of differentially accumulated proteins from the secondary leaves of cowpea plants according to their biological functions. Plants inoculated with CPSMV at 2 (A) and 6 (B) DAI; Plants submitted to drought at 2 (C) and 6 (D) DAI; and submitted to the combined stress at 2 (E) and 6 (F) DAI.

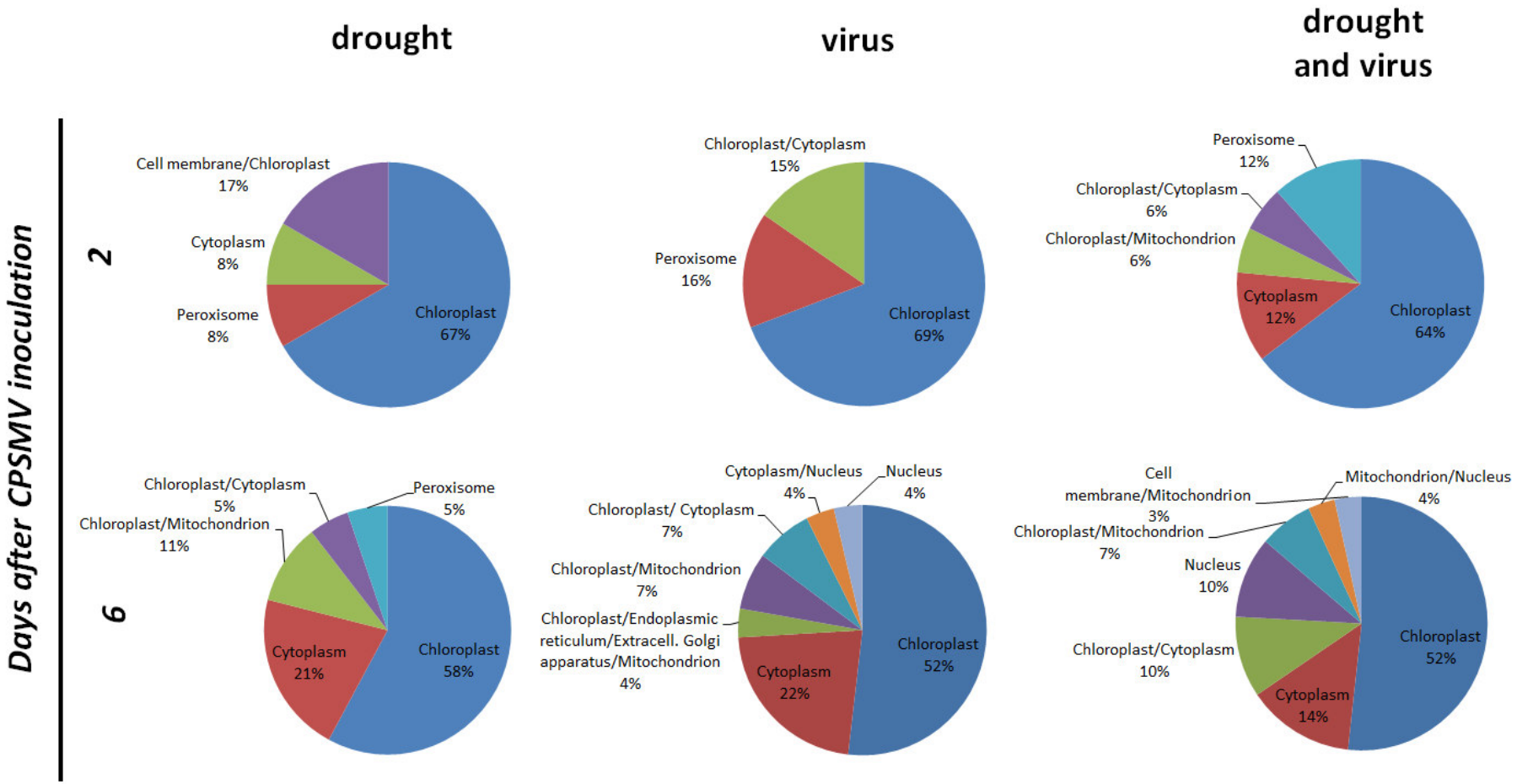

Fig. 3. Subcellular localization of proteins from the secondary leaves of cowpea plants exposed to drought, infected with CPSMV, and submitted to the combined stresses (DV).

4 Discussion

Proteins from the secondary leaves of cowpea plants that changed in abundance under drought stress, when infected with CPSMV, and submitted to the combined stresses have been identified, using label-free proteomic approach (LC-MS/MS). These proteins are clustered in nine major groups based on their putative biological functions (Tab. 1, Fig. 2). Six groups out of nine are emphasized below because they encompass the higher protein number.

4.1 Energy metabolism and photosynthesis

In the present study, most of the differentially accumulated proteins are involved in energy metabolism and photosynthesis pathways (Tab. 1, Fig. 2) suggesting the importance of these processes in response to abiotic and biotic stresses.

ATP synthase, an enzyme responsible for ATP production, was up-represented in all groups (virus, drought, and the combined drought stress and virus-infection) 2 days after CPSMV inoculation (Table 1). Likewise, ATP synthase was induced in tobacco under drought stress (Gharechahi *et al*., 2015) and tomato plants infected with *Potato virus X* (PVX) (Cueto-Ginzo *et al*., 2016). Energy metabolism seems to be an integrative part of the regulatory mechanisms to produce the energy that is needed to efficiently combat stress conditions. However, at 6 DAI, most proteins involved with energy metabolism and photosynthesis were down-represented in the plant groups only under drought stress (D), only infected with CPSMV (V), and also at 2 DAI when both stresses were combined (DV). This can be important as a plant strategy for energy conservation.

Fructose-bisphosphate aldolase is an enzyme involved in multiple pathways including glycolysis, gluconeogenesis, and the Calvin cycle. In higher plants, different isoforms of this enzyme are located in the cytoplasm, where they are involved with the glycolytic pathway, or in the chloroplast, where they participate in the Calvin cycle (Razdan *et al*., 1992; Lebherz *et al*., 1984). In our study, a fructose-bisphosphate aldolase from chloroplast was up-represented in

cowpea plants under drought and under the combined drought stress and CPSMV-infection, but not only virus-infected, at 2 DAI, suggesting that this enzyme responded to drought stress and has a role in the adaption of cowpea to this condition.

Photosynthesis involves the light and Calvin cycle (dark) reactions. Light-dependent reactions of photosynthesis occur at the thylakoid membrane where four major protein complexes, named Photosystem II (PSII), Cytochrome b6f complex, Photosystem I (PSI), and ATP synthase, participate. Several proteins that play essential roles in both reactions have negative effects in viral pathogenesis (Li *et al.*, 2016). In this current study, many photosynthetic proteins were down-represented in the CPSMV-infected (such as PS I reaction center subunit II chloroplastic-like protein and PS I subunit VII) and drought-stressed (PS II CP43 chlorophyll apoprotein) cowpea plants at 2 DAI, as well as in the plant group under the combined stresses at 6 DAI (PS I reaction center subunit III, PS II CP47 and CP43 chlorophyll apoprotein) (Tab. 1, Fig. 2). It has been reported that the viral coat protein (CP) accumulation can reduce the PSII activity leading to the photoxidation of chloroplasts in infected cells and to symptom development (Reinero and Beachy, 1989; Hodgson *et al.*, 1989). Regarding to drought, it is known that this stress inhibits the photosynthetic activity because the imbalance between light capture and its utilization (Reddy *et al.*, 2004). Similarly, Zadražnik *et al.* (2013) also observed, after proteomic analysis, decrease in the abundance of proteins involved with photosynthesis in common bean (*Phaseolus vulgaris* L., cv Starozagorski) under water stress.

Oxygen evolving enhancer protein 1 (OEE1) was identified (up-represented) among the proteins that exhibited changes in abundance profile in cowpea plants subjected to CPSMV infection and those submitted to the combined stresses (Tab. 1). OEE1 is a manganese-stabilizing protein that belongs to the oxygen-evolving complex of photosystem II required for PSII core assembly/stability (Yi *et al.*, 2005). In addition, it has been reported that photosystem II oxygen-evolving complex protein interacts with the coat protein of *Alfalfa mosaic virus* and inhibits its replication in *Nicotiana benthamiana* (Balasubramaniam *et al.*, 2014). Therefore, in the present study, OEE1 accumulation in cowpea plants infected with CPSMV suggests a plant attempt to avoid CPSMV replication.

4.2 Cell redox homeostasis

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) play important roles in cell signaling throughout the kingdoms (Bailly, 2004; Laloi *et al.*, 2004; D'Autre´aux and Toledano, 2007). In plants, H_2O_2 is a major signaling molecule involved in various biological processes such as regulation of growth and development, and responses to biotic and abiotic stresses (Mittler *et al.*, 2011). However, excess hydrogen peroxide can provoke cytotoxic effect leading to cellular damage and subsequent cellular death. Thus, plants require systems to regulate ROS levels to maintain the cell redox homeostasis. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and others play important roles in plant cells, protecting them against the deleterious effects of ROS (Caverzan *et al.*, 2012; Wu *et al.*, 2006).

Peroxisomal catalase is important for the removal of H_2O_2 produced during photorespiration (Noctor *et al.*, 2000). In the present study, this protein was down-represented in all groups (virus, drought, and the combined stresses) at 2 DAI (Tab. 1). Under severe drought stress the activity of antioxidant enzymes are inhibited and/or down-represented if the peroxide levels are high (Selote and Khanna-Chopra, 2010). In regard to virus infection, it was reported that *CAT3* overexpression in transgenic plants diminished the *Cucumber mosaic virus* (CMV) accumulation whereas the CMV 2b protein (a known RNA-silencing suppressor), by directly interacting with the enzyme CAT3 (protein-protein interaction), neutralizes the host CAT3 cellular activity of scavenging cellular H_2O_2 and favored CMV infection, leading to virus-induced specific necrosis (Inaba *et al.*, 2011). Thus, according to these above findings, it is concluded that the decrease of catalase activity seems to favor both abiotic and biotic stresses.

Peroxirredoxin (Prx) is a group of H_2O_2-decomposing antioxidant enzymes. In this study, one Prx was up-represented in cowpea plants infected with CPSMV (2 DAI) and submitted to drought and CPSMV in combination, at 2 and 6 DAI (Tab. 1). Hakmaoui *et al.* (2012) observed that plants infected with the less virulent strain of Pepper Mild Mottle Virus (PMMoV-I) maintained the

levels of three Prxs. This result suggests that this enzyme plays a role in response to viral infection in an attempt to maintain the H_2O_2 levels.

Copper/zinc superoxide dismutase (Cu/Zn-SOD), an H_2O_2-generating enzyme, was up-represented only in the cowpea plant group in which the combined drought stress and virus-infection were imposed, at 6 DAI (Tab. 1). *Nicotiana benthamiana* infected with PMMoV-I showed a higher capacity to eliminate superoxide radical (O_2^-) due to higher activities of all SOD isoenzymes (Hakmaoui *et al.*, 2012). As in our study Cu/Zn-SOD was not up-represented in drought stresses neither in CPSMV-infected plants, but only when these two stresses were combined, it seems that the combined stresses might evolve higher superoxide generation and thus Cu/Zn-SOD up-accumulation to maintain redox homeostasis.

4.3 Protein Folding & Metabolism

Cyclophilins are a family of ubiquitous proteins (Galat, 1999) that have peptidyl-prolyl isomerase activity and catalyze the *cis/trans* isomerization of peptide bonds at proline residues and hence protein folding (Brandts *et al.*, 1975). It was previously reported that cyclophilin interact with viral RNA and replication proteins inhibiting virus multiplication (Mendu *et al.*, 2010; Lin *et al.*, 2012; Kovalev and Nagy, 2013). In our study, cyclophilin was up-regulated in plants at 6 DAI (Tab. 1), probably as an attempt to avoid, unsuccessfully, CPSMV multiplication in cowpea plants.

4.4 Regulation factors or RNA processing

The success of viral infection depends on the interaction between virus components and host factors (Ahlquist *et al.*, 2003; Nagy and Pogany, 2012). The eukaryotic elongation factors (eEFs), amongst them the universally conserved eukaryotic elongation factor 1A (in short eEF1A, formerly EF-Tu) and 1B (in short eEF1B, formerly EF-Ts) are necessary for translation elongation in eukaryotes because they deliver amino acyl tRNA (aa-tRNA) to the ribosome in a codon-specific manner (Le Sourd *et al.*, 2006, Taylor *et al.*, 2006; Marintchev and Wagner, 2004). In our study, eEF1A and eEF1B were up-represented in

plants inoculated both with CPSMV and submitted to the combined drought stress and virus-infection, respectively, at 6 DAI. These findings reinforce the importance of eEF1A and eEF1B for viral replication. Indeed, the interaction between eEF1A and the 3' untranslated region (3' UTR) of *Turnip yellow mosaic virus* (TYMV) RNA increased TYMV RNA translation (Matsuda and Dreher, 2004).

4.5 Response to stress

Heat shock proteins (HSPs) are induced in cells exposed to different stresses. They are involved in a variety of biological processes such as folding of newly synthesized polypeptides, refolding of aggregated proteins, protein complex assembly or disassembly, translocation of proteins, and others (Whitley *et al.*, 1999). HSPs are classified into family groups in accordance with the molecular weight: HSP110, HSP90, HSP70/HSP80, HSP60, and small molecular HSP (smHSP) (Tecsi *et al.*, 1994).

HSPs play important roles in defense against abiotic stresses such as drought (Sato and Yokoya, 2008) protecting proteins, lipids, nucleic acids and cytoskeleton (Tkacova and Angelovicova, 2012). Viral infections can also induce HSPs. Studies indicate that several viruses recruit HSP90 chaperone that functions to assist in the synthesis, maturation and stabilization of viral proteins (Young *et al.*, 2001). On the other hand, in tobacco, HSP90 interacts with the resistance protein N and its signaling proteins SGT1 and Rar1 to modulate an innate immune response (Liu *et al.*, 2004). In this current study, four HSPs were indentified (Tab. 1). None of them were detected in the cowpea plants submitted only to the drought stress (D group), suggesting that they did not respond to this artificial imposed condition. Two HSPs (PREDICTED: stromal 70 kDa heat shock-related protein, chloroplastic-like and Heat Shock 70kD protein) were up-represented in both the CPSMV-infected (V) and the cowpea plants submitted to the combined drought stress and CPSMV-infection (DV) at 2 DAI and also at 6 DAI. Perhaps up-representation of this two specific HSPs is more linked to the CPSMV infection itself, which consequently is also verified when the cowpea plants were submitted to the combined stresses. Noticeably, other two HSPs (17.7 kDa class I small heat shock protein and heat

shock protein 90-2) were up-represented exclusively in the cowpea plants submitted to the combined stresses (DV) at 6 DAI, but not at 2 DAI. Therefore two hypotheses arise. (1) response of these two particular HSPs is associated solely with the drought stress imposed to the cowpea plants, but not with CPSMV-infection; (2) or they are responsive to the CPSMV infection itself, but in a scenario in which the cowpea plants became more susceptible to the virus infection caused by the imposition of the drought stress. In agreement with the first hypothesis, Sato and Yokoya (2008) found that overproduction of the small heat shock protein 17.7 increased drought tolerance in transgenic rice seedlings. Corroborating with the second one, it was suggested that HSP90 could act both facilitating (Young *et al.*, 2001) and compromising (Liu *et al.*, 2004) viral infection.

4.6 Protein–protein interaction analysis of identified proteins

In living organisms, proteins do not act independently. They form a variety of interactions which are essential for biological processes (Miernyk and Thelen, 2008). In the present work, protein interaction network created revealed functional links between different proteins. For all experimental cowpea plant groups, two major clusters of interaction, in which proteins involved in photosynthesis and those involved in energy & metabolism (marked with circles), were discerned (Fig. 4, 5 and 6), highlighting the fundamental importance of these proteins and the associated metabolic processes in the response of cowpea to the stresses imposed.

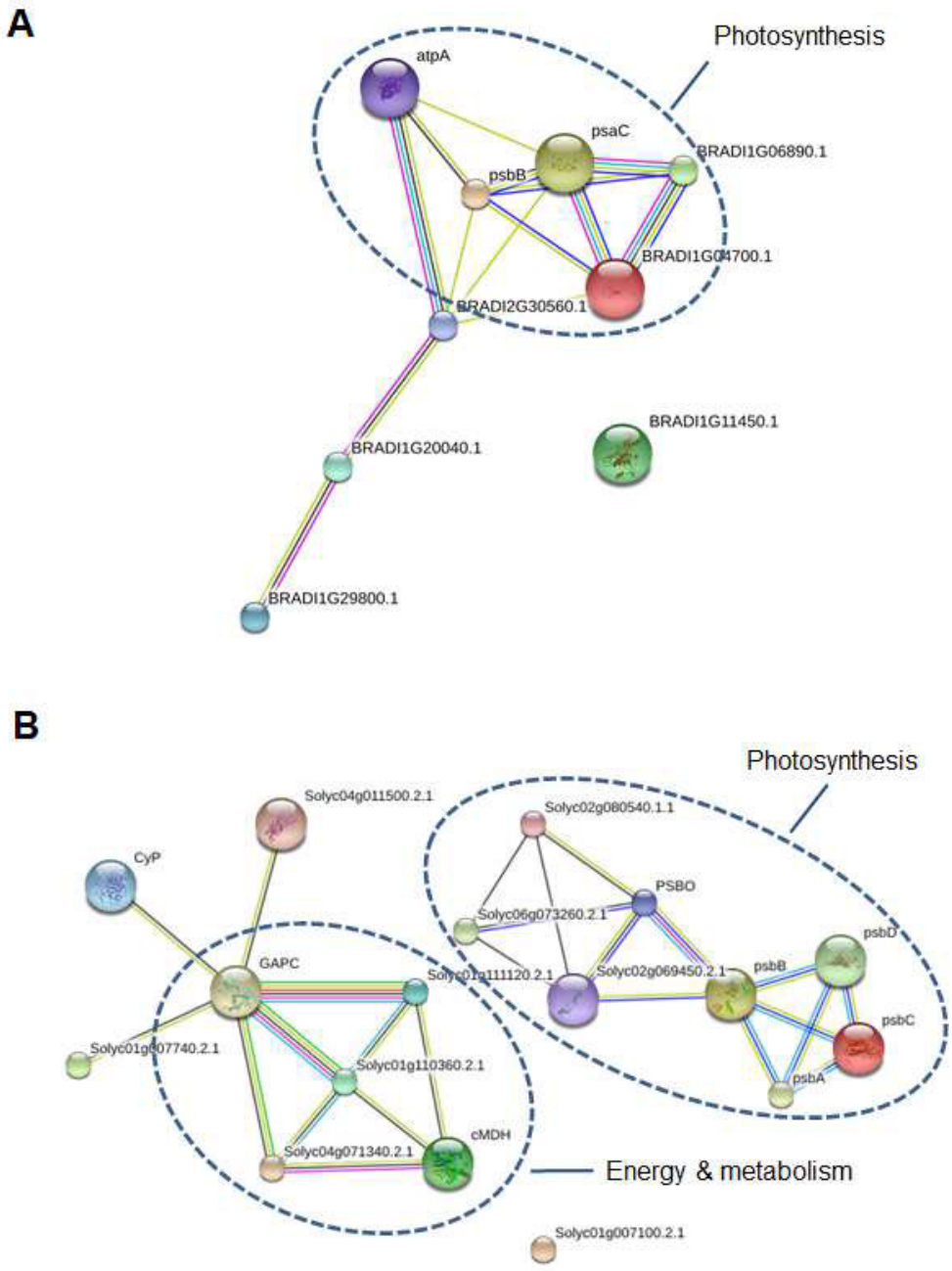

Fig. 4. Interaction network (String software) of differentially represented proteins in the cowpea secondary leaves (CE-31 genotype) submitted to CPSMV infection at 2 (A) and 6 (B) days after CPSMV inoculation. Different colored lines indicate the types of evidence for the associations: green line, neighborhood evidence; redline, fusion evidence; blue line, co-occurrence evidence; black line, coexpression evidence; purple line, experimental evidence; light blue line, database evidence; yellow line, text-mining evidence, and light purple line, homology evidence. Abbreviations of the specific protein names in the network *ar*e given in Supplementary Table S1.

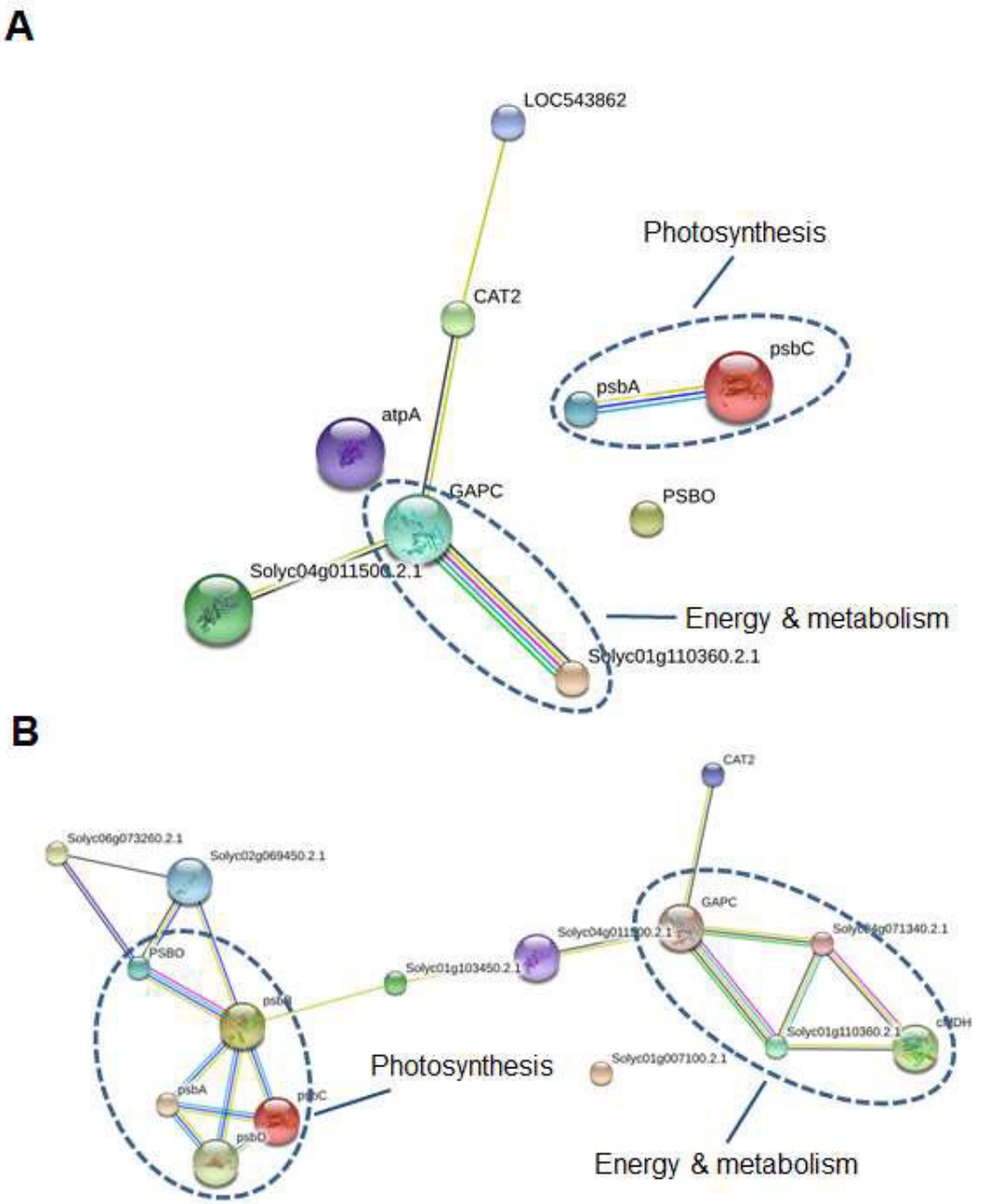

Fig. 5. Interaction network (String software) of differentially represented proteins in the cowpea leaves (genotype CE-31) submitted to drought stress (25% pot capacity) at 2 (A) and 6 (B) days after CPSMV inoculation. Different colored lines indicate the types of evidence for the associations: green line, neighborhood evidence; redline, fusion evidence; blue line, co-occurrence evidence; black line, coexpression evidence; purple line, experimental evidence; light blue line, database evidence; yellow line, text-mining evidence, and light purple line, homology evidence. Abbreviations of the specific protein names in the network *ar*e given in Supplementary Table S1.

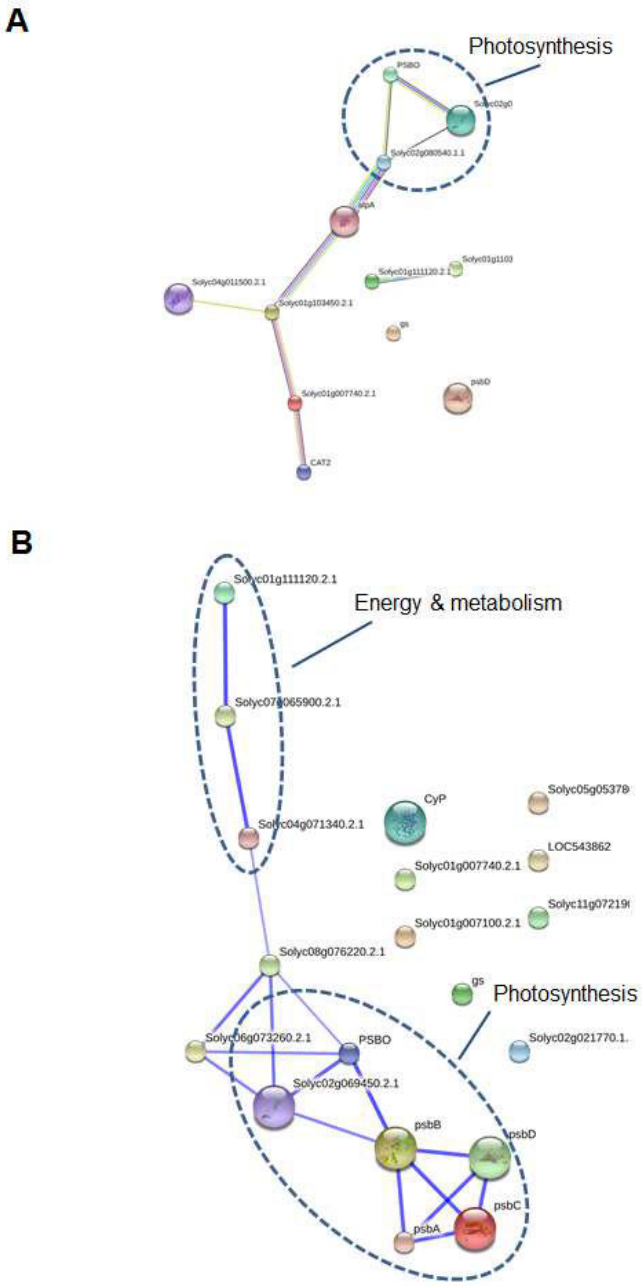

Fig. 6. Interaction network (String software) of differentially represented proteins in the cowpea secondary leaves (CE-31 genotype) submitted to the combined drought stress (25% pot capacity) and CPSMV-infection at 2 (A) and 6 (B) days after CPSMV inoculation. Different colored lines indicate the types of evidence for the associations: green line, neighborhood evidence; redline, fusion evidence; blue line, co-occurrence evidence; black line, coexpression evidence; purple line, experimental evidence; light blue line, database evidence; yellow line, text-mining evidence, and light purple line, homology evidence. Abbreviations of the specific protein names in the network *a*re given in Supplementary Table S1.

5 Conclusions

In the CPSMV-inoculated cowpea plants, down-representation of proteins involved with energy/metabolism and photosynthesis, at 6 DAI, appears to be important for the viral disease establishment and thus cowpea susceptibility. Down-representation of photosynthesis proteins in cowpea plants under drought is consistent with the photosynthetic limitation imposed by this kind of stress. When submitted to the combined drought stress (25% pot capacity) and CPSMV-infection cowpea plants recruited various proteins at 6 DAI. All together these findings are important for understanding the molecular mechanisms behind the responses of a CPSMV susceptible cowpea genotype (CE-31) infected with this virus and subjected to drought, alone or in combination.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Silva, R. G. G. acknowledges a Doctoral grant from Coordination for the Improvement of Higher Education Personnel (CAPES), the Brazilian National Council for Scientific and Technological Development (CNPq) and Ceara State Research Foundation (FUNCAP).

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Supplementary information

Table S1 - Abbreviations of the specific protein names in the protein-protein networks (STRING 10.0)

Abbreviation	**Protein name**
Energy & Metabolism	
atpA	ATP synthase CF1 alpha subunit
Solyc01g110360.2.1	probable fructose-bisphosphate aldolase 2
Solyc02g080540.1.1	ATP synthase gamma chain
Solyc04g071340.2.1	fructose-1,6-bisphosphatase
cMDH	malate dehydrogenase
GAPC	glyceraldehyde-3-phosphate dehydrogenase
Solyc01g111120.2.1	triosephosphate isomerase
LOC543862	vacuolar H+-ATPase subunit A
Solyc07g065900.2.1	fructose-bisphosphate aldolase 1
Solyc08g076220.2.1	phosphoribulokinase
Photosynthesis	
BRADI1G04700.1	photosystem I reaction center subunit II chloroplastic-like protein
BRADI1G06890.1	photosystem I reaction center subunit III
psaC	photosystem I subunit VII
psbA	photosystem II protein D1
psbB	photosystem II CP47 chlorophyll apoprotein
psbC	photosystem II CP43 chlorophyll apoprotein
psbD	photosystem II protein D2
Solyc02g069450.2.1	photosystem I reaction center subunit III
PSBO	oxygen-evolving enhancer protein 1
Cell redox homeostasis	
BRADI1G29800.1	Catalase
Solyc01g007740.2.1	Peroxiredoxin
CAT2	Catalase
Protein Folding & Metabolism	
BRADI1G11450.1	GLUTAMINE SYNTHETASE
BRADI1G20040.1	Peroxiredoxin

CyP	peptidyl-prolyl cis-trans isomerase
Gs	glutamine synthetase precursor

Regulation factors or RNA processing	
Solyc06g073260.2.1	chloroplast stem-loop binding protein of 41 kDa a
Solyc11g072190.1.1	elongation factor 1 beta

Response to stress	
BRADI2G30560.1	stromal 70 kDa heat shock-related protein
Solyc01g103450.2.1	stromal 70 kDa heat shock-related protein
Solyc05g053780.2.1	glycine-rich RNA-binding protein

Lipid biosynthesis	
Solyc01g007100.2.1	DIGALACTOSYLDIACYLGLYCEROL SYNTHASE 1

Structural Protein	
Solyc04g011500.2.1	Actin

CONSIDERAÇÕES FINAIS

Neste trabalho foi demonstrado que o estresse hídrico aumentou a susceptibilidade do feijão-de-corda (CE-31) ao CPSMV no estágio inicial da infecção (2 DAI) e que a infecção viral não agravou efeitos da deficiência hídrica. O estudo proteômico revelou que o feijão-de-corda responde ativamente a esses estresses (individualmente ou simultaneamente impostos) por meio de reprogramação gênica, levando a alterações no perfil proteômico. Estes resultados confirmam que as respostas da planta aos estresses individuais são diferentes de quando os estresses são simultaneamente impostos e, esta segunda condição, aumenta a agressividade dos sintomas da doença.

Todos os resultados em conjunto são importantes para a compreensão dos mecanismos moleculares das respostas de um genótipo susceptível de feijão-de-corda (CE-31) infectado com o CPSMV e submetido a seca, ou combinação desses estresses.

ANEXO

Experimento I - areia como substrato

Aspecto visual de plantas e folhas de feijão-de-corda (cv. CE-31) sob deficiência hídrica e/ou inoculação com CPSMV

Após dois dias da inoculação do CPSMV, o substrato (areia) já estava completamente desidratado. Dessa forma, as plantas já apresentavam sintomas característicos da deficiência hídrica (murchamento das folhas) (FIGURA 1). Com 4 dias após inoculação do CPSMV, as plantas submetidas ao estresse hídrico já estavam em senescência (FIGURA 1). Portanto, a areia utilizada no experimento não seria uma boa alternativa para impor a deficiência hídrica, pois esse substrato não possui boa capacidade de retenção hídrica para que o estresse fosse gradativo e mais prolongado.

Figura 01. Imagem representativa de plantas de feijão-de-corda (cv. CE-31) após suspensão de rega e/ou inoculação com CPSMV nos tempos de 2 e 4 dias após inoculação do vírus.

A Figura 2A mostra as folhas após 4 dias da inoculação do CPSMV e deficiência hídrica. As folhas infectadas pelo vírus apresentaram sintomas de

infecção e aquelas que foram submetidas, concomitantemente, ao estresse biótico e abiótico apresentaram-se mais debilitadas. Após 7 dias da inoculação do CPSMV, a infecção tornou-se sistêmica, pois foram observados sintomas da doença nas folhas secundárias (FIGURA 2B).

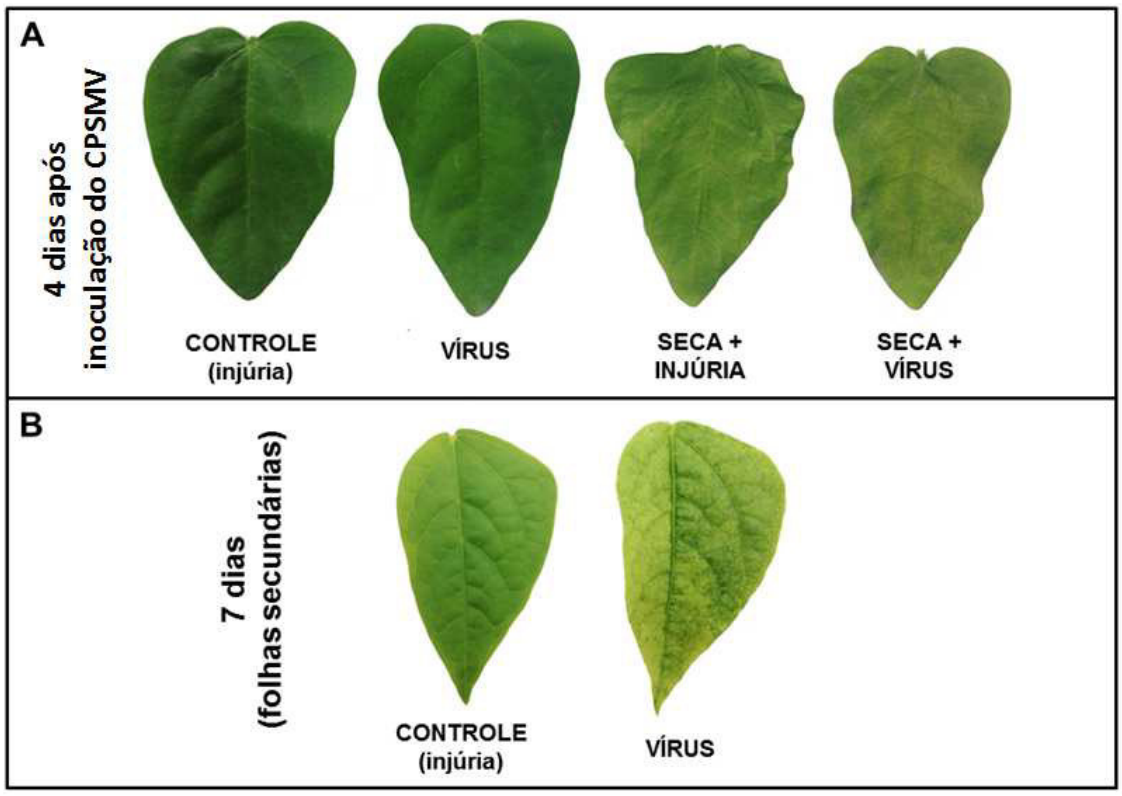

Figura 02. Imagem representativa de folhas de feijão-de-corda (cv. CE-31) após suspensão de rega e/ou inoculação do CPSMV nos tempos de 2 e 4 dias após inoculação do vírus (A). Folhas secundárias de feijão-de-corda (cv. CE-31) após 7 dias da inoculação com CPSMV (B).

Parâmetros fisiológicos (trocas gasosas e fluorescência da clorofila a)

A deficiência hídrica promoveu uma drástica diminuição nos parâmetros relacionados a trocas gasosas e fluorescência da clorofila a após 2 dias da inoculação do CPSMV, em comparação ao controle (FIGURA 3). PN, E e gS apresentaram redução de 100% em relação ao controle no último dia de experimento nas plantas submetidas ao estresse hídrico (FIGURA 3A, B e C). A eficiência quântica do fotossistema II (Fv/Fm) foi diminuída apenas no último

dia de experimento para as plantas com déficit hídrico, sendo essa diminuição maior nas plantas submetidas aos dois estresses (hídrico e infecção pelo vírus) (Figura 3D). A infecção pelo vírus causou uma diminuição em PN e E a partir de 72 horas do início do experimento em relação ao controle (FIGURA 3A e B). Já para gS, foi observada uma diminuição a partir de 24 horas em relação ao controle (FIGURA 3D).

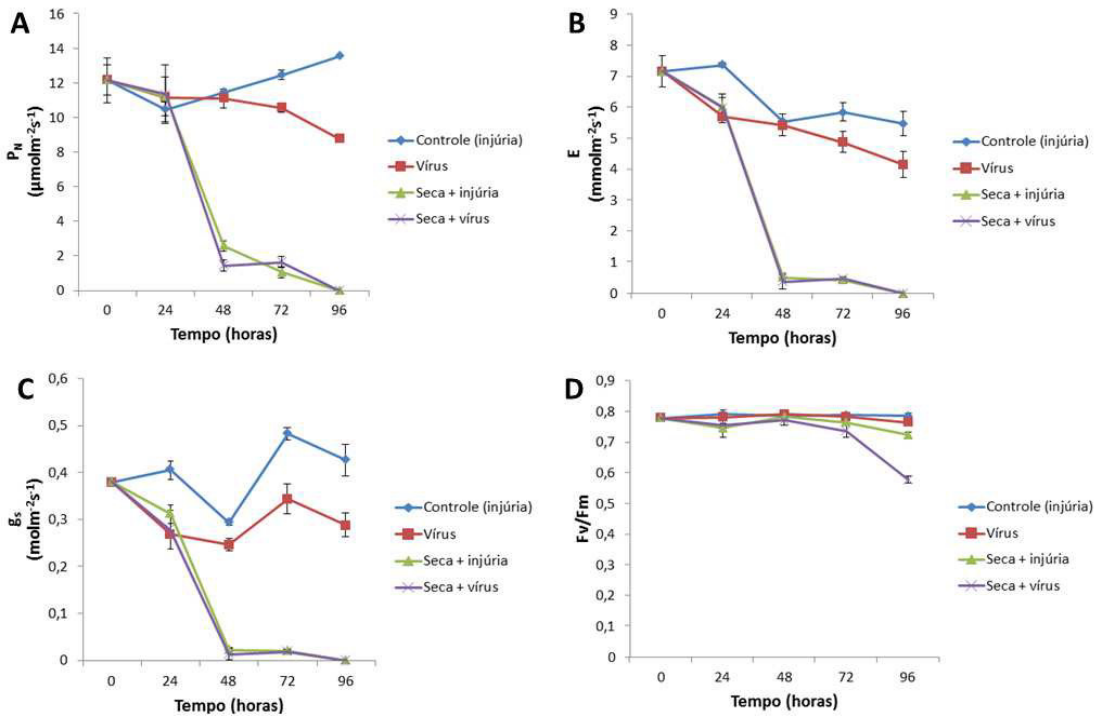

Figura 03. Fotossíntese líquida (A), transpiração (B), condutância estomática (C) e eficiência quântica potencial do fotossistema II (D) em folhas de feijão-de-corda (cv. CE-31) após suspensão de rega e/ou inoculação com o CPSMV nos tempos de 2 e 4 após inoculação do vírus.

Experimento II - vermiculita/areia como substrato

A avaliação da perda de água ao longo do dia no substrato vermiculita/areia, na proporção 1:1 (m/m), revelou diminuição de 10% e 4% na massa da água no substrato saturado com 100% e 25% da capacidade de campo, respectivamente (Figura 5). Devido o substrato areia/vermiculita, na

proporção 1:1 (m/m) ter demonstrado forte capacidade de retenção de água, a proporção de areia foi aumentada para uma nova avaliação ao longo de 7 dias.

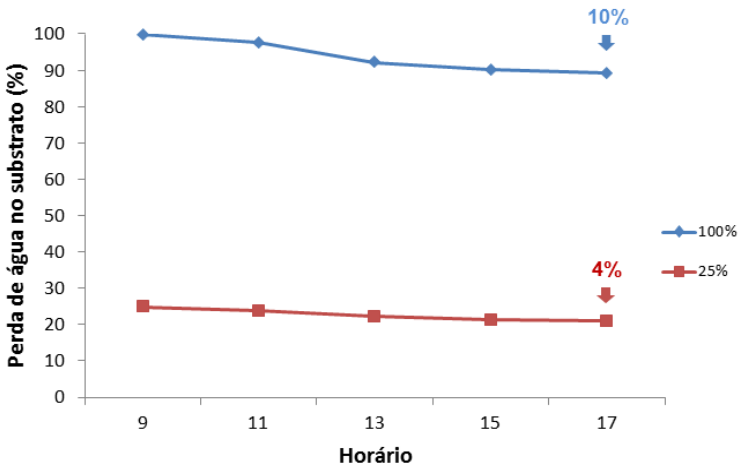

Figura 05. Perda de água em substrato vermiculita/areia proporção 1:1 (p/p) ao longo do dia em vasos dispostos em casa de vegetação. 100% da capacidade de campo; 25% da capacidade de campo.

A Figura 6A mostra que a diminuição na massa da água foi maior para o substrato vermiculita/areia, na proporção 1:9 (m/m), em comparação a vermiculita/areia na proporção 1:1 (m/m), revelando que esse substrato seria adequado para a realização de um novo experimento. A perda de água no substrato vermiculita/areia, proporção 1:1 (m/m), saturado com 50% da capacidade de campo foi baixa ao longo de sete dias (Figura 6B). A figura 6C mostra que a perda de água no substrato com planta de feijão-de-corda transplantada não apresentou diferença em relação ao substrato sem planta ao longo de sete dias.

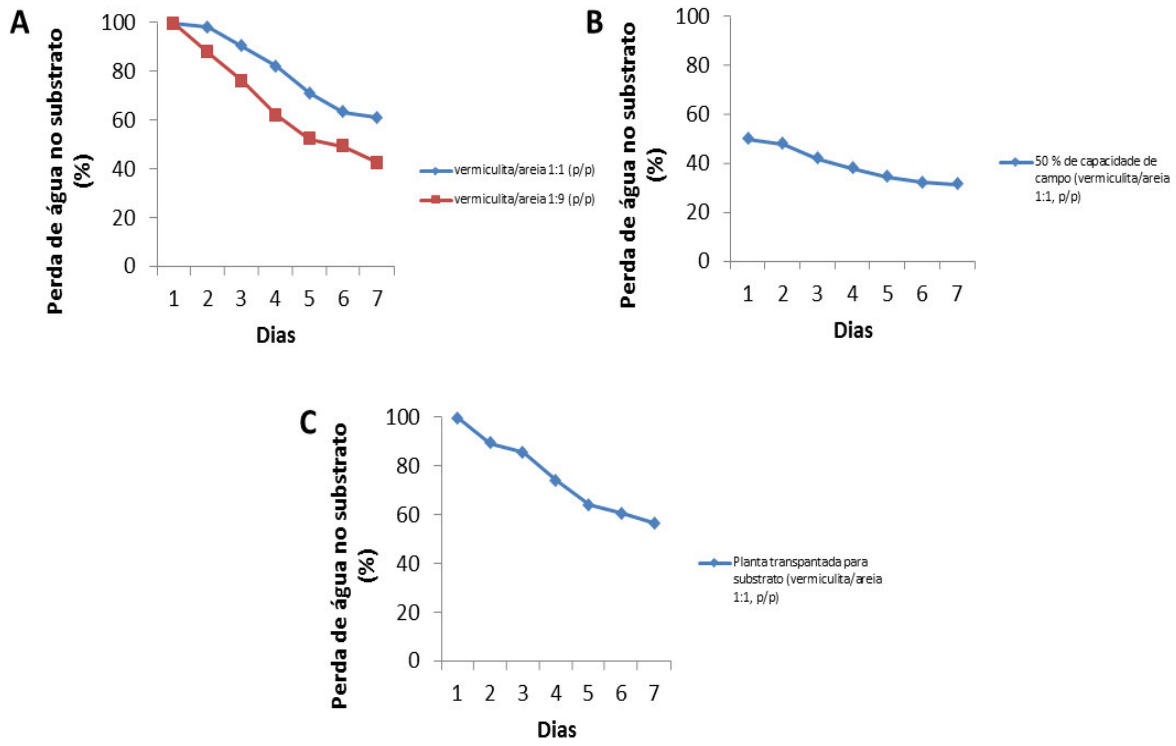

Figura 06. Percentual de água em vermiculita/areia 1:1 (p/p) e vermiculita/areia 1:9 (p/p) (A), vermiculita/areia 1:1 (p/p) com 50% de capacidade de campo (B) e vermiculita/areia 1:1 (p/p) com planta de feijão-de-corda transplantada para o substrato (C) durante 7 dias em casa de vegetação. Em A e C o substrato foi saturado no início do experimento com 100% da capacidade de campo.

O crescimento do feijão-de-corda (cv. CE-31) em vermiculita/areia, proporção 1:9 (m/m) apresentou-se da mesma forma em comparação à planta crescida apenas em areia (Figura 7). Assim, foi constatado que o substrato vermiculita/areia, na proporção 1:9 (m/m), é adequado para o cultivo de feijão-de-corda (cv. CE-31) nas condições preconizadas para nossos experimentos.

Figura 07. Imagem representativa de plantas de feijão-de-corda (cv. CE-31) cultivadas em vermiculita/areia 1:9 (p/p) e somente areia após 10 dias de transplantio.

Com o objetivo de verificar quantos dias demorariam para o substrato passar de um percentual de capacidade de campo de 70% para 25% (deficiência hídrica), a rega de plantas de feijão-de-corda com 10 dias foi suspendida (Figura 8A). A Figura 8B revelou que quatro dias após suspender a rega, o substrato vermiculita/areia, proporção 1:9 (p/p) passa de 70% de capacidade de campo para 25%.

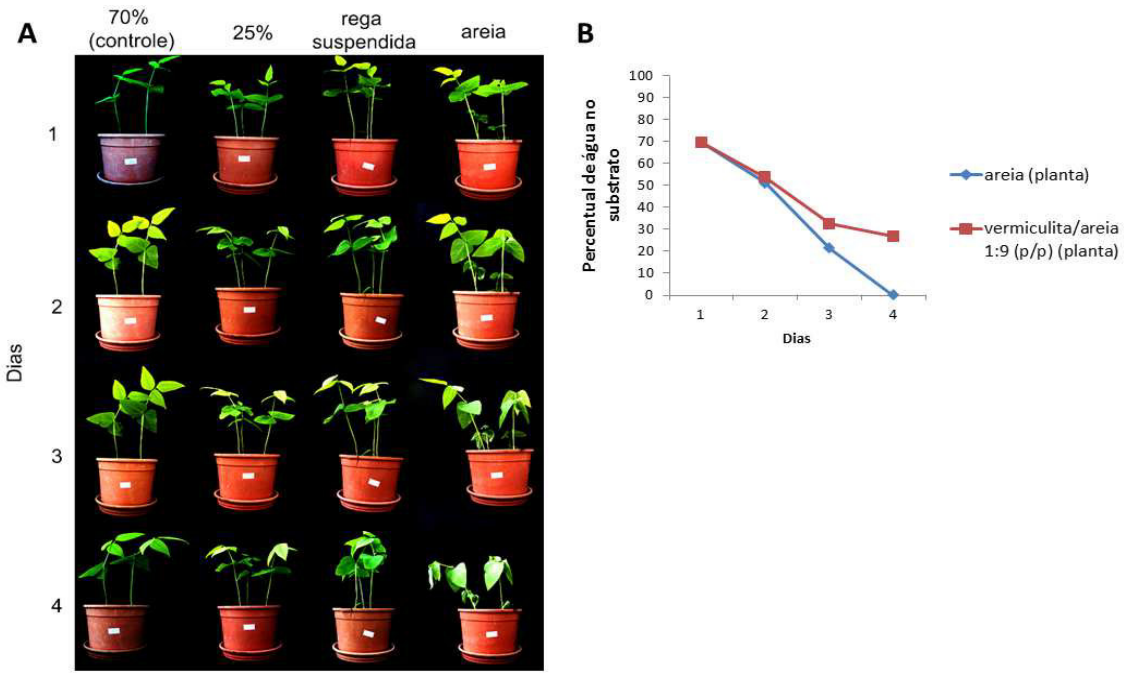

Figura 08. Imagem representativa de plantas de feijão-de-corda (*Vigna unguiculata*) com 10 dias em casa de vegetação submetidas aos seguintes tratamentos: 70% e 25% de capacidade de campo, rega suspendida e rega suspendida em areia (A). Percentual de água nos substratos (B).