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PEDRO FILHO NORONHA DE SOUZA

ANÁLISE COMPARATIVA DE PARÂMETROS BIOQUÍMICOS E FISIOLÓGICOS DE UM GENÓTIPO DE FEIJÃO-DE-CORDA (*Vigna unguiculata* L. Walp.) SUSCETÍVEL E SEU DERIVADO MUTAGENIZADO RESISTENTE AMBOS INFECTADOS COM O *VÍRUS DO MOSAICO SEVERO DO CAUPI* (CPSMV)

FORTALEZA – CEARÁ
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Tese apresentada à coordenação do Programa de Pós-Graduação em Bioquímica como requisito obrigatório para a obtenção do título de Doutor em Bioquímica pela Universidade Federal do Ceará.

Orientador: Prof. Dr. José Tadeu Abreu de Oliveira

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Do not worry about your difficulties in Mathematics.

I can assure you mine are still greater.

Albert Einstein (1879-1955)

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RESUMO

O feijão-de-corda tem grande importância socioeconômica no Nordeste brasileiro. Entretanto, sua produção é baixa devido a diversos fatores bióticos, como, por exemplo, o vírus do mosaico severo do caupí (CPSMV, gênero *Comovirus*), que apresenta grande destaque, por causar a virose que mais acomete essa cultura no país. No estudo da interação planta-vírus, diversos trabalhos mostram que o tratamento de sementes com o etil metanosulfonato (EMS, mutagênico químico) resulta no fenótipo de resistência em plantas que, anteriormente, apresentavam susceptibilidade à infecção por vírus do gênero *Potyvirus*. Por essa razão, o presente estudo teve como objetivo investigar as respostas de defesa bioquímicas e fisiológicas das plantas de feijão-de-corda do genótipo (CE-31) susceptível ao CPSMV (CPI) a partir de sementes tratadas com EMS (0,04% v/v), e avaliar se as plantas mutagenizadas (MCPI), produzidas a partir dessas sementes se tornaram resistentes ao CPSMV. Duas diferentes abordagens foram utilizadas neste trabalho: 1) análises bioquímicas (enzimas antioxidantes e conteúdo de H_2O_2, PR-proteínas e compostos secundários) e fisiológicas (parâmetros fotossintéticos e teor de clorofila); 2) abordagem proteômica quantitativa (LC-ESI-MS/MS), livre de marcação, para identificar proteínas responsivas à infecção viral. Os resultados obtidos demonstram que as plantas MCPI são capazes de induzir respostas bioquímicas (aumento de H_2O_2, indução de PR-proteínas e aumento no conteúdo de compostos secundários) e alterações nos parâmetros fisiológicos (alta taxa fotossintética e teor de clorofila) que, aparentemente, têm relação com o fenótipo de resistência das plantas mutagenizadas ao CPSMV. Na análise proteômica, 99 proteínas foram identificadas como sendo diferenciais, das quais 68 aumentaram e 31 diminuíram em abundância nas plantas MCPI em relação as plantas CPI. A análise proteômica, mostrou diversas vias metabólicas (Metabolismo Redox, Energia e Metabolismo, Fotossíntese, Metabolismo de RNA e Defesa) envolvidas nas respostas de defesa das plantas MCPI frente a infecção viral. O tratamento das sementes com o EMS, resultou em plantas de feijão-de-corda com fenótipo de resistência capazes de acionar mecanismos de defesa para impedir a infeção viral.

Palavras chaves: Feijão-de-corda, EMS, CPSMV, mecanismos de defesa de plantas, LC-ESI-MS/MS

ABSTRACT

Cowpea is an important crop that makes major nutritional contributions as a source of proteins and carbohydrates in the diet of many people worldwide. However, its production is impaired due to various stresses including those of biotic origins. Cowpea Severe Mosaic Virus (CPSMV) infects cowpeas leading to severe symptoms and low productivity. Several studies of plant-virus interaction show that seed treatment with Ethyl methanesulfonate (EMS, chemical mutagen), results in a resistant phenotype in plants, which was previously susceptibility, to virus infection of the *Potyvirus* genus. The aim of this study was to investigate some physiological and biochemical parameters of a susceptible cowpea cultivar (CPI) (CE-31, sin. Pitiuba) in comparison with its derived resistant mutagenized (MCPI), both infected with CPSMV. MCPI plantlets were obtained after treatment of CE-31 seeds with 0.04% EMS. Two different approaches were used in this study: 1) biochemical (antioxidant enzymes and H_2O_2 content, PR-proteins and secondary metabolites) and physiological analysis (photosynthetic parameters and chlorophyll content); and 2) Label free quantitative proteomic approach (LC-ESI-MS / MS) to identify proteins responsive to viral infection. Our results showed that MCPI had no symptoms of CPSMV infection and biochemical (high H_2O_2, PR-proteins and secondary compounds [phenolic and lignin]) and physiological responses (High photosynthesis index and chlorophyll content) is activated in MCPI plantlets after CPSMV inoculation. With regard to proteomic analysis, 99 proteins were differentially represented, where these 68 are up- and 31 down represented in MCPI compared to CPI. Regardless whether to CPI (susceptible) or MCPI (mutagenized resistant) plantlets, CPSMV induce changes in proteome profile that involve several biological process (energy and metabolism, photosynthesis, response to stress, oxidative burst, and scavenging). Moreover, these results suggest that the CPSMV responsive proteins in the MCPI represent a complex network involving in resistant mechanisms to CPSMV. Treatment of the susceptible CE-31 genotype seeds with the mutagenic agent EMS induced genomic alterations generating a cowpea mutagenized resistant to CPSMV by apparently inducing classical biochemical and physiological responses against infection.

Keywords: Cowpea, EMS, CPSMV, plant defense, LC-ESI-MS/M

LISTA DE ABREVIATURAS

∆F/Fm'	Eficiência quântica efetiva do fotossistema II
BICMV	Vírus do mosaico do caupi, variedade "blackeye"
CABMV	Vírus do mosaico do caupi transmitido pelo pulgão
CCMV	Vírus do mosqueado clorótico do caupi
CDPK	Proteína quinase dependente de cálcio
CGMV	Vírus do mosaico dourado do caupi
Ci	Pressão interna de CO_2
CMV	Vírus do Mosaico do Pepino
CONAB	Companhia Nacional de Abastecimento
CPI	Plantas de feijão-de-corda obtidas a partir de sementes não tratadas com EMS e inoculadas com o CPSMV.
CPMMV	Vírus do mosqueado do caupi
CPMV	Vírus do mosaico do caupí
CPSMV	Vírus do mosaico severo do caupi
CPU	Grupo de plantas do genótipo CE-31, suscetíveis, inoculado apenas com tampão fosfato de potássio 10 mM, pH 7 + carborundum, nao infectado com o CPSMV (controle)
DAB	3´3 – Diaminobenzidina
DAMPS	Padrões Moleculares Associados aos Danos
dsRNA	Double-Stranded RNA
eEF	Fatores de Elongação Eucariótico
eIF	Fatores de Iniciação da Tradução Eucariótico
EST	Etiquetas de Sequências Transcritas
ETI	Imunidade Desencadeada por Efetores
FAO	Organização para a Agricultura e Alimentação
FV/FM	Eficiência quântica máxima do fotossistema II
gs	Condutância estômatica
HR	Resposta hipersensitiva
ICVT	Comitê Internacional de Taxonomia Viral
MAMPS	Padrões Moleculares Associados aos Microrganismos
MAPK	MAP quinase

MCPI	Grupo de plantas mutagenizada com EMS, resistentes, infectadas com o CPSMV
miRNA	MicroRNA
NBS-LRR	Proteína ligante a nucleotíde com sequências repetidas de resíduos de leucina
NPQ	Quenching não-fotoquímico
PAMPS	Padrões Moleculares Associados aos Patógenos
PCD	Morte Celular Programada
PMSF	Fluoreto de Fetilmenilsufonil (Inibidor de protease Serínica)
P_N	Fotossíntese Liquída
PPFD	Densidade de Fluxo de fótons fotossíntetico
PR-proteínas	Proteínas Relacionadas à Patogênese
PRR	Receptores de Reconhecimento de Padrões
PTGS	Silenciamento Gênico Pós-Transcricional
PTI	Imunidade Desencadeada por PAMPs
PVPP	Polivinil Polipirrolidona
PVX	Vírus da Batata X
Qp	Quenching fotoquímico
RISC	Complexo de Silenciamento Induzido Por RNA
RNAi	Silenciamento por RNA de interferência
RNS	Espécies Reativas de Nitrogênio
ROS	Espécies Reativas de Oxigênio
RT-PCR	Reação de Amplificação Semi-Quantitativa pela RNA polimerase de Transcritos Reversos
RT-qPCR	Amplificação Quantitativa de Transcritos Reversos
SAR	Resposta Sistêmica Adquirida
EMS	Etil metanosulfonato
siRNA	Pequeno RNA de intereferência
sRNA	Pequeno RNA
TGS	Silenciamento Gênico Transcricional
TMV	Vírus do Mosaíco do Tabaco
TNV	Vírus do Mosaico do Nabo
VAMPS	Padrões Moleculares Associados ao Vírus

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CAPÍTULO I

FUNDAMENTAÇÃO TÉORICA

Pedro Filho Noronha de Souza

1. Fundamentação Teórica

1.1 Feijão-de-corda

O feijão-de-corda [*Vigna unguiculata* (L.) Walp.], também conhecido como feijão-caupi, é uma planta dicotiledônea, pertencente à ordem *Fabales*, família *Fabaceae*, subfamília *Faboideae*, tribo *Phaseoleae*, subtribo *Phaseolinae*, gênero *Vigna,* espécie *Vigna unguiculata*. Sua origem é confusa, contudo, evidências sugerem que essa cultura tenha se originado no continente africano e introduzida nas Américas pelos espanhóis, que realizavam o tráfego de escravos. No Brasil, é encontrada, predominantemente, nas regiões Norte e Nordeste, onde é considerado o principal alimento das famílias residentes nessas regiões (EHLERS; HALL, 1997).

O feijão-de-corda é um produto da horticultura tradicional comum, sendo uma das culturas de leguminosas mais importantes em regiões tropicais. Do ponto de vista nutricional, o feijão-de-corda apresenta alto teor proteico, carboidratos, fibras alimentares, vitaminas e minerais (Ca, Mg, Na, K e Fe), além de possuir maior quantidade de ácidos graxos insaturados frente aos saturados. Na dieta, o feijão-de-corda, além de aumentar a quantidade de proteínas ingeridas, também contribui para melhorar qualidade dessas proteínas (PHILIPS *et al.,* 2003; VASCONCELOS *et al.,* 2010; ABREU *et al.,* 2012; WAWIRE et al., 2012).

O feijão-de-corda é uma espécie que possui boa capacidade de fixação do nitrogênio, conferindo-lhe boa adaptação a solos pobres, com pouca água e altas temperaturas (20-35 °C) (EHLERS; HALL, 1997). Essas características fazem das regiões Norte e Nordeste do Brasil um ótimo ambiente para o crescimento e desenvolvimento dessa cultura, embora, também, possa crescer bem em outras regiões. O Brasil está entre os maiores produtores de feijão-de-corda do mundo, com produção de 3.320,400 toneladas em 2014. Nesse contexto, o Estado do Ceará contribuiu com uma produção de 132.500 toneladas no ano de 2014 gerando emprego e renda para milhares de pessoas (SILVA, 2012; CONAB, 2014; FAO, 2014).

Apesar de ser adaptada a diversos tipos de estresses abióticos e bióticos, a cultura de feijão-de-corda é, constantemente, desafiada pelo ataque de patógenos

(vírus, bactérias, fungos e nematoides) que causam danos à integridade das plantas e perdas irreparáveis, que afetam, diretamente, a produção e a comercialização das sementes, resultando em prejuízos para economia (NEVES *et al.,* 2011). Dentre todos os patógenos citados, os vírus representam o principal grupo de patógenos que afetam a produtividade do feijão-de-corda.

Existem oito espécies de vírus, que são patógenos do feijão-de-corda, sendo distribuídas em seis famílias distintas: o Vírus do mosaico do caupí (CPMV, *Comovirus*) (FAN *et al.,* 2011); Vírus do mosaico severo do caupi (CPSMV, *Comovirus*) (ABREU *et al.,* 2012); Vírus do mosaico do caupi transmitido pelo pulgão (CABMV, *Potyvirus*) (BARROS *et al.,* 2011); Vírus do mosaico do caupi, variedade "blackeye" (BICMV, *Potyvirus*) (SHILPASHREE, 2006); Vírus do mosaico dourado do caupi (CGMV, *Germivirus*) (JOHN *et al.,* 2008); Vírus do mosaico do pepino (CMV, *Cucumovirus*) (HUA *et al.,* 2012); Vírus do mosqueado clorótico do caupi (CCMV, *Bromovirus*) (ALI; ROOSSINCK 2008); e Vírus do mosqueado do caupi (CPMMV, *Carmovirus*) (BRITO *et al.,* 2012). Dentre todos os vírus citados, o de maior ocorrência no Brasil é o CPSMV.

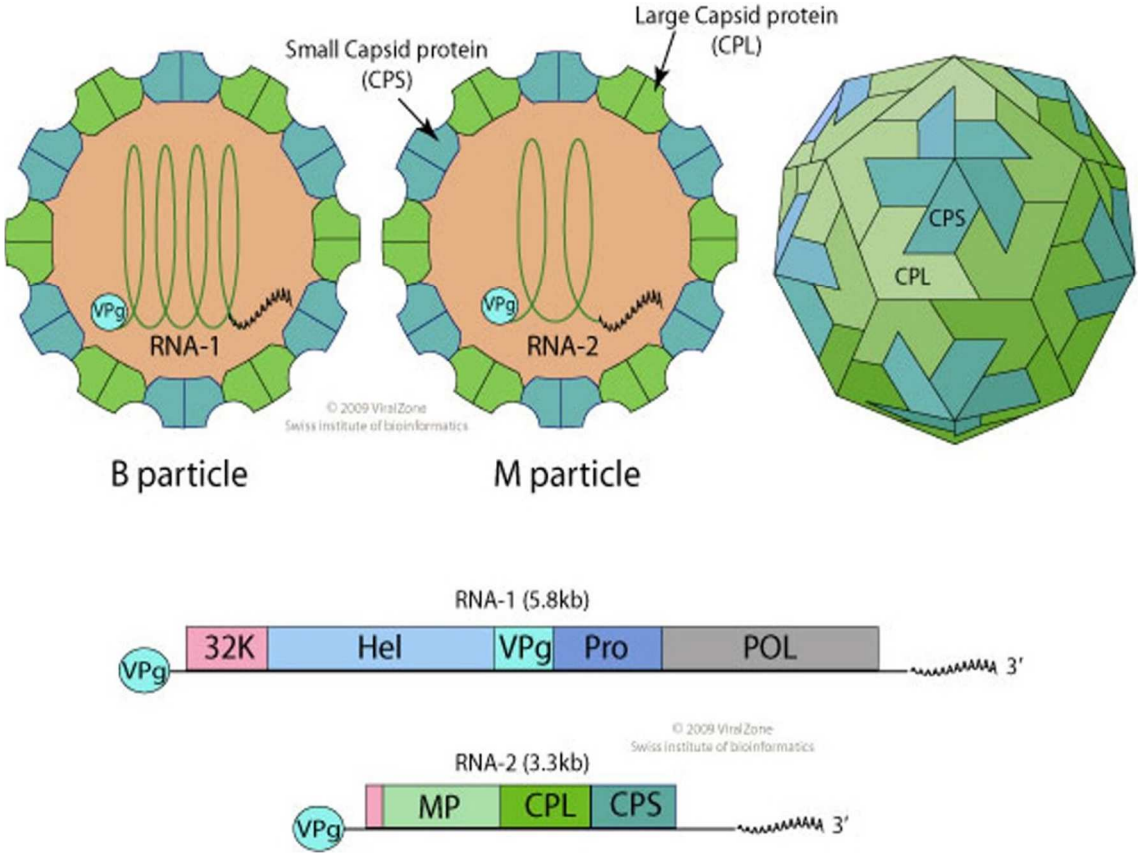
1.2 - Vírus do mosaico severo do caupi

O vírus do mosaico severo do caupi (CPSMV) pertence à ordem *Picornavirales*, família *Secoviridae*, subfamília *Comovirinae*, gênero *Comovirus*, espécie *Vírus do mosaico severo do caupi* (*Cowpea Severe Mosaic Virus,* Intenational Committe on Taxonomy of Viruses, 2012 - ICTV). O CPSMV é um vírus que possui como material genético uma molécula de RNA (retrovírus) bipartido, constituído pelo RNA 1 (também chamado de RNA-B ou *bottom component*) e RNA 2 (também chamado RNA-M ou *Middle component*) sendo ambos de polaridade positiva (+) (CHEN; BRUENING, 1992a).

As duas moléculas de RNA são encapsuladas separadamente em partículas icosaédricas de 28 nm de diâmetro cada. Ambas as fitas do RNA viral possuem uma proteína denominada VPg (do Inglês, *Viral Genome-linked Protein*) covalentemente ligada à extremidade 5´, além de serem poliadeniladas na extremidade 3´ (**Figura 1**) (CHEN: BRUENING, 1992a). VPg atua tanto como um iniciador (primer), após a proteína ser uridilada, fornecendo um grupo hidroxílico livre que pode ser estendido pela RNA-polimerase viral dependente de RNA, bem como tem papel na iniciação da

tradução agindo como 5' mRNA cap. O RNA-1 (5´→3´) codifica para uma poliproteína que, após modificações pós-traducionais, resulta em cinco proteínas maduras importantes para a replicação viral, sendo estas, uma protease, um cofator da protease, uma helicase (Hel), uma proteína VPg e uma RNA polimerase RNA-dependente (CHEN; BRUENING, 1992b; FAN *et al.,* 2011).

Figura 1 - Representação gráfica do capsídeo composto por proteínas da capa e genoma bipartido (RNA 1 e RNA 2) do CPSMV. 32K - uma protease; PRO - um cofator da protease; HEL – helicase; VPg - proteína VPg; POL - RNA polimerase RNA-dependente; MP – Proteína de movimento; CPL – proteína da capa viral alta peso molecular; e CPS - proteína da capa viral baixo peso molecular.

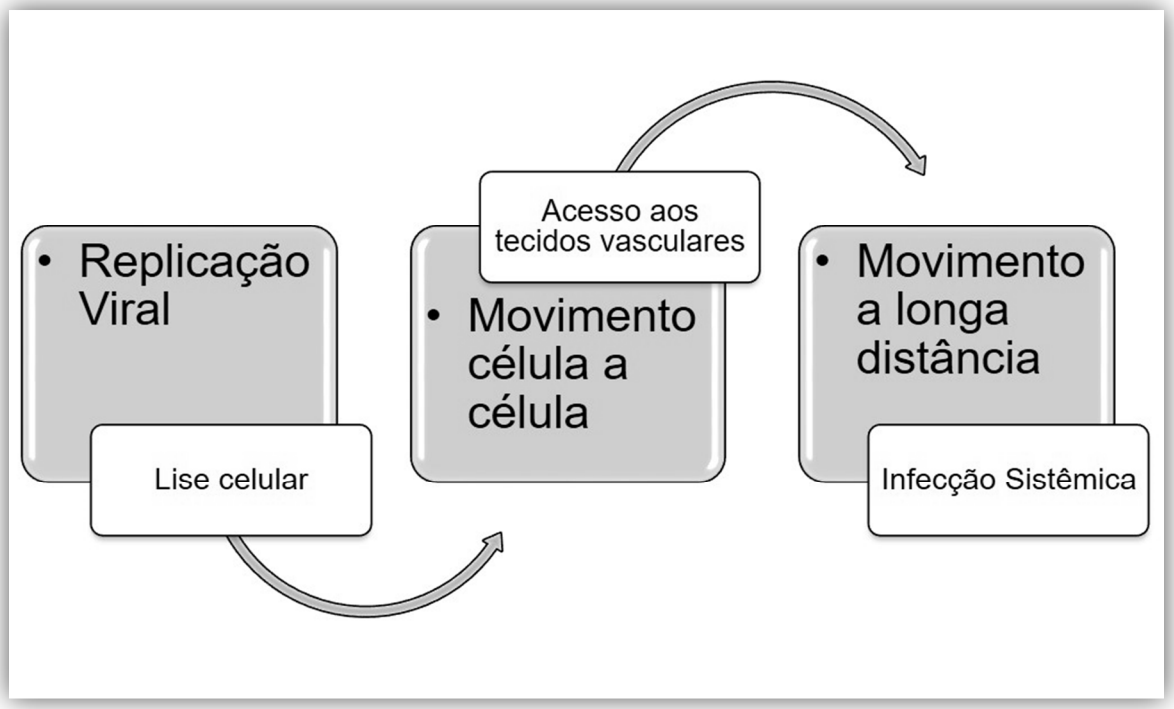


Fonte: http://viralzone.expasy.org/all_by_species/728.html

O RNA 2 é composto por 1244 pares de bases e codifica uma poliproteína de 1002 resíduos de aminoácidos, composta por uma proteína de movimento célula a célula e duas subunidades proteicas, uma maior (~41 kDa) e uma menor (~21 kDa),

constituintes da capa viral (CHEN; BRUENING, 1992b; LOMONOSSOF; GHABRIAL, 2001). Essas proteínas são importantes no momento da infecção viral, sendo utilizadas durante a interação inicial do vírus com a planta, como no caso da proteína da capa viral, e a segunda, é uma proteína de movimento, sendo importante no movimento do vírus célula a célula e movimento sistêmico (WEBER; BUJARSKI, 2015) (**Figura 2**). Uma vez que o vírus infecta uma célula, ele pode se espalhar por todo o tecido através do movimento célula a célula via plasmodesmas, e, ao alcançar os tecidos vasculares, onde pode se movimentar para tecidos distantes do sítio de infecção primário, resultando em doença sistêmica (CARRINGTON *et al.,* 1996; NELSON; VAN BEL, 1998).

Figura 2 – Movimentação do vírus nas plantas infectadas.

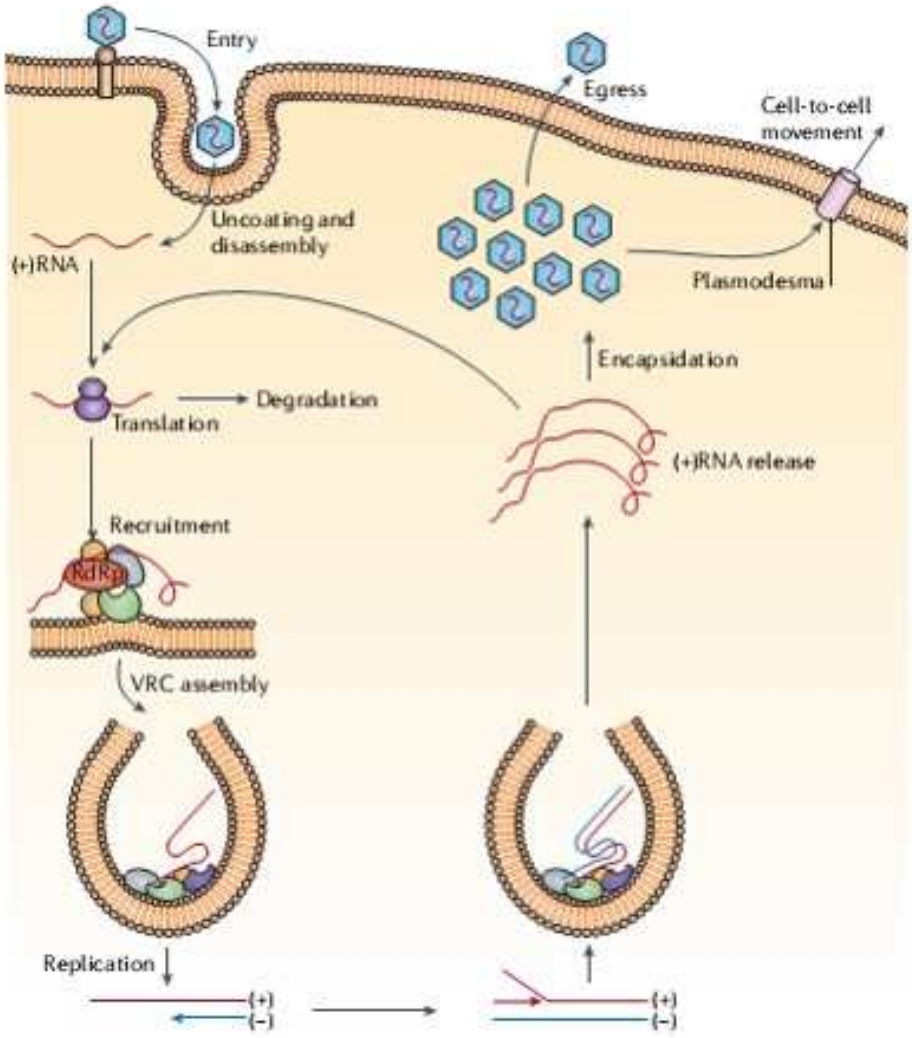


Fonte: Autor.

A replicação do CPSMV segue o mesmo princípio dos vírus de RNA de sentido positivo (+) que infectam plantas. Quando o vírus penetra na célula, o genoma viral é colocado para fora do capsídeo icosaédrico. Sua replicação tem como passo inicial a replicação do RNA (+) produzindo uma fita de RNA complementar (-). Simultaneamente a esse processo, a RNA polimerase RNA-dependente utiliza a fita complementar (-) como molde para a produção de inúmeras outras cópias do RNA

(+) (LALIBERTÉ; SANFAÇON, 2010). A VPg ligada na porção 5´do RNA viral permite sua interação com os ribossomos da célula iniciando a síntese das proteínas virais. Para se defender das ribonucleases da célula, o CPSMV possui um mecanismo que também ocorre em outros membros do Gênero *Comovirus*, no qual são induzidas invaginações de membrana chamada de VRC (do inglês, *Viral Replication Complex*) formando vesículas que protegem o genoma viral de ribonucleases da planta, permitindo, assim, a continuidade da sua replicação (**Figura 3**) (CARRETTE *et al.,* 2000; NAGY; POGANY, 2012).

Figura 3 – Modelo esquemático da interação do vírus com a membrana da e entrada do vírus na célula, seguida da formação de vesículas virais que favorecem a replicação viral.



Fonte: NAGY;POGANY, 2012.

Sendo o repertório gênico viral muito pequeno, devido ao tamanho limitado do genoma, os vírus necessitam recrutar proteínas do hospedeiro para completar o ciclo de replicação e, também, para fugir das defesas da planta. Além dos ribossomos utilizados para traduzir o RNA viral, fatores de iniciação da tradução (eIF4G e eIF4E), importantes para ligação do RNA viral aos ribossomos, e fatores de elongação 1 (eEF1A e eEF1B), importantes no processo de elongação da proteína nascente, são exemplos de proteínas do hospedeiro que favorecem a replicação viral. Os fatores eEF1 e eIF4 são importantes em diversos processos celulares, como metabolismo de proteínas (síntese e degradação), movimentação celular, transporte nuclear, dentre outros. Sendo, o eEF1 (A e B) e o eIF4 (G e E) proteínas multifuncionais, não é surpresa que os vírus utilizem essas proteínas para facilitar seu processo de replicação (HWANG *et al*., 2013 e 2015; LI *et al*., 2015; SANFAÇON, 2015).

O primeiro relato da incidência do CPSMV no Nordeste foi feito por Lima e Nelson (1977) e, desde então, diversos estudos têm indicado a variabilidade dos isolados de CPSMV (BESERRA-Jr *et al.,* 2011). A sintomatologia da doença apresenta-se no limbo foliar, e os principais sintomas são bolhosidades, manchas cloróticas, mosaico severo (**Figura 4**), lesões necróticas locais, subdesenvolvimento das nervuras principais, distorção foliar, alterações no tamanho e no formato do limbo, e, quando as plantas infectadas são jovens, apresentam nanismo. As sementes se apresentam deformadas e, também, com baixa taxa de germinação (ZERBINI; CARVALHO; MACIEL-ZAMBOLIN*,* 2002). O mosaico severo está entre as mais importantes doenças do feijão-de-corda no Brasil, pois ocorre em todas as regiões onde existem plantações dessa cultura, causando perdas na produtividade. Dependendo do cultivar e do tempo de inoculação, o CPSMV pode reduzir em até 80% o rendimento das plantações de feijão-de-corda (BROOKER; UMAHARAN; McDAVID, 2005; LIMA; SITTOLIN; LIMA, 2005).

Figura 4 – Sintomas da doença 4 (**A**) e 7 (**B**) dias após infeção do feijão-de-corda genótipo CE-31pelo CPSMV.

Fonte: Autor.

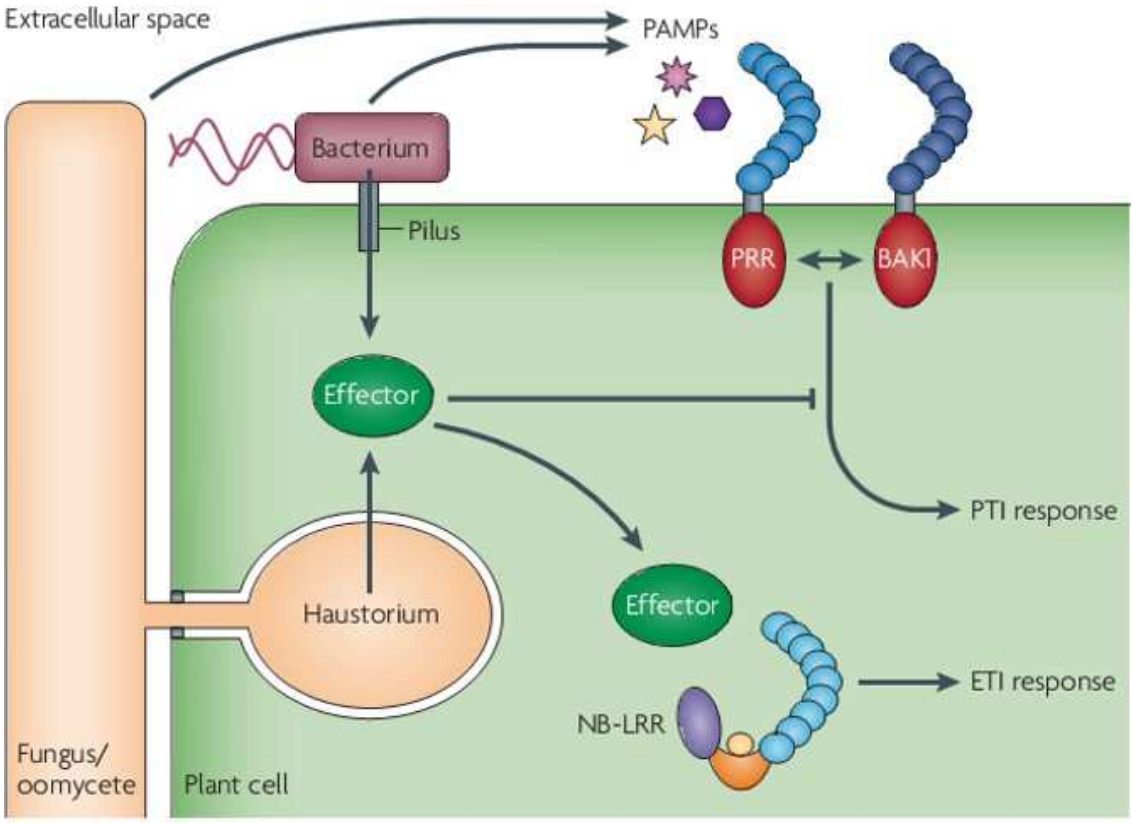
Não existe ainda uma forma de controle eficaz, que previna e/ou combata essa doença. Atualmente, o controle do CPSMV é realizado apenas pelo cultivo de genótipos de feijão-de-corda que são resistentes ou imunes ao vírus, como, por exemplo, o Macaibo que é originado da coleção de germoplasma do Instituto Agronômico de Pernambuco (IPA), e foi identificado como sendo imune ao CPSMV (CAMARÇO *et al.,* 2009). No caso do genótipo Macaibo, sua resistência é associada a uma característica monogênica recessiva (resistência recessiva). Porém, a utilização de cultivares resistentes para evitar a doença causada pelo CPSMV pode, também, não ser uma alternativa viável, pois, Lima e colaboradores (2012) demonstraram a existência de um isolado de CPSMV que é capaz de infectar o genótipo Macaibo, onde ele se desenvolve, causando os mesmos sintomas severos da doença. Devido sua capacidade de infectar o genótipo Macaibo esse isolado foi denominado de CPSMV-MC.

1.3 Mecanismos de Defesa de Planta contra patógenos

Os mecanismos de defesa das plantas são divididos em duas fases: a PTI (do inglês *Pathogen-Triggered Immunity*), sendo a primeira linha de defesa vegetal ou resistência basal e a ETI (do inglês *Effector-Triggered Immunity*) ou resistência induzida por efetores, constituindo a segunda linha de defesa das plantas (JONES; DANGL, 2006; MUTHAMILARASAN; PRASAD 2013). Após o contato com o patógeno, a PTI pode ser rapidamente ativada pelo reconhecimento extracelular dos MAMPS ou PAMPs (do Inglês, *Microbe-Associated Molecular Patterns* ou *Pathogen-Associated Molecular Patterns*), DAMPs (do inglês, *Damage-associated Molecular Patterns*), e/ou VAMPs (do inglês *Viral-Associate Molecular Patterns*), que são padrões moleculares conservados, necessários para a sobrevivência dos patógenos como, por exemplo, a flagelina das bactérias, haustório de fungos e dsRNA (do inglês, *Double strand RNA*), sendo reconhecidos por receptores PRR (do inglês, *Pattern Recognition Receptors*) ou BAK1 (do inglês, *Brassinosteroid Insensitive 1-Associated Kinase*) (**Figura 5**) (SOOSAAR *et al*., 2005; DODDS; HALTER *et al*., 2014; HOHN, 2013; NICAISE; ROUX; ZIPFEL, 2009; PUMPLIN; VOINNET, 2013).

Os PRRs são proteínas que possuem na sua estrutura um domínio receptor extracelular, um domínio transmembrana que mantém a proteína ancorada à membrana plasmática, e um domínio citoplasmático com função de quinase, necessário para desencadear a sinalização das respostas de defesa no interior da célula. Após reconhecimento dos padrões moleculares pelos PRRs, diversos eventos são induzidos na tentativa da planta de prevenir infecção. Esses eventos incluem a sinalização mediada por íons Ca^{2+} e a explosão oxidativa, mediada por espécies reativas de oxigênio (ROS, do inglês, *Reactive Oxygen Species*) e espécies reativas de nitrogênio (RNS, do inglês, *Reactive Nitrogen Species*), bem como ativação de diferentes proteínas tais como MAPK (do inglês, *Mitogen-Activated Protein Kinase*) e CDPK (do inglês, *Calcium-dependent Protein Kinases*), que induzem reprogramação da expressão de PR-genes (do inglês, *Pathogenesis-Related Genes*) (NICAISE; ROUX; ZIPFEL, 2009; STOTZ *et al*., 2013).

Figura 5 - Reconhecimento de PAMPs e indução de resposta imune da planta (PTI), seguida de supressão induzida por efetores dos patógenos. Após efetores dos patógenos suprimirem a PTI plantas induzem a expressão de ETI, uma resposta direcionada com a ação do efetor do patógeno .



FONTE: DODDS; RATHJEN, 2010

Essas respostas das plantas mostram que a interação entre hospedeiro e o patógeno é dinâmica e sugerem que a PTI seja decorrente da coevolução entre plantas e patógenos, durante a “corrida armamentista” desses organismos. Para tentar sobrepujar a PTI, ou seja, essa primeira linha de defesa das plantas, os patógenos, ao longo da evolução, adquiriram a capacidade de produzir moléculas efetoras, supressoras da PTI, denominadas Avr (do Inglês, *Avirulence Proteins*). Por sua vez, as plantas coevoluíram e passaram a sintetizar proteínas denominadas de NBS-LRR (do inglês, *Nucleotide Binding site Leucine-Rich Repeat*), ou Proteínas R (**Figura 5**), que podem reconhecer essas moléculas efetoras do patógeno, ou seja, as proteínas Avr, neutralizando sua ação (JONES; DANGL, 2006; DODDS; RATHJEN, 2010; COLL; EPPLE; DANGL, 2011). As proteínas R são codificadas por

genes R do hospedeiro e, ao reconhecerem as proteínas efetoras do patógeno, desencadeiam a imunidade acionada pelos efetores ou ETI (do Inglês, *Effector-Triggered Immunity*), caracterizando a segunda linha de defesa vegetal (CARR; LEWSEY; PALUKAITS, 2010; STIRNWEIS *et al*., 2014; CHEN *et al*., 2016; SARRIS *et al*., 2016).

Esse mecanismo de reconhecimento de proteínas Avr do patógeno por proteínas R do hospedeiro foi proposto, pela primeira vez, por Flor (1971), sendo denominado hipótese gene-a-gene. Essa hipótese preconiza que, para que a planta seja resistente a um patógeno específico, existe a necessidade de reconhecimento do produto do gene Avr do patógeno pelo produto do gene de resistência (gene R) do hospedeiro (**Figura 6A**).

Outra forma de interação entre as proteínas Avr e as proteínas R é chamada de hipótese-guarda, que é uma variação da hipótese gene-a-gene. A hipótese guarda implica no reconhecimento indireto de efetores do patógeno pelas proteínas R (*Guard Protein*) (**Figura 6B**), através do monitoramento da integridade de proteínas celulares (*Guardee Protein*) que são alvos diretos dos efetores dos patógenos. Após a percepção de alterações causados nessas proteínas, devido a interação com proteínas Avr do patógeno, processos de sinalização celular, que resultam na ativação dos mecanismos de defesa, são acionados (JONES; DANGL 2006; SOOSAAR *et al*., 2005).

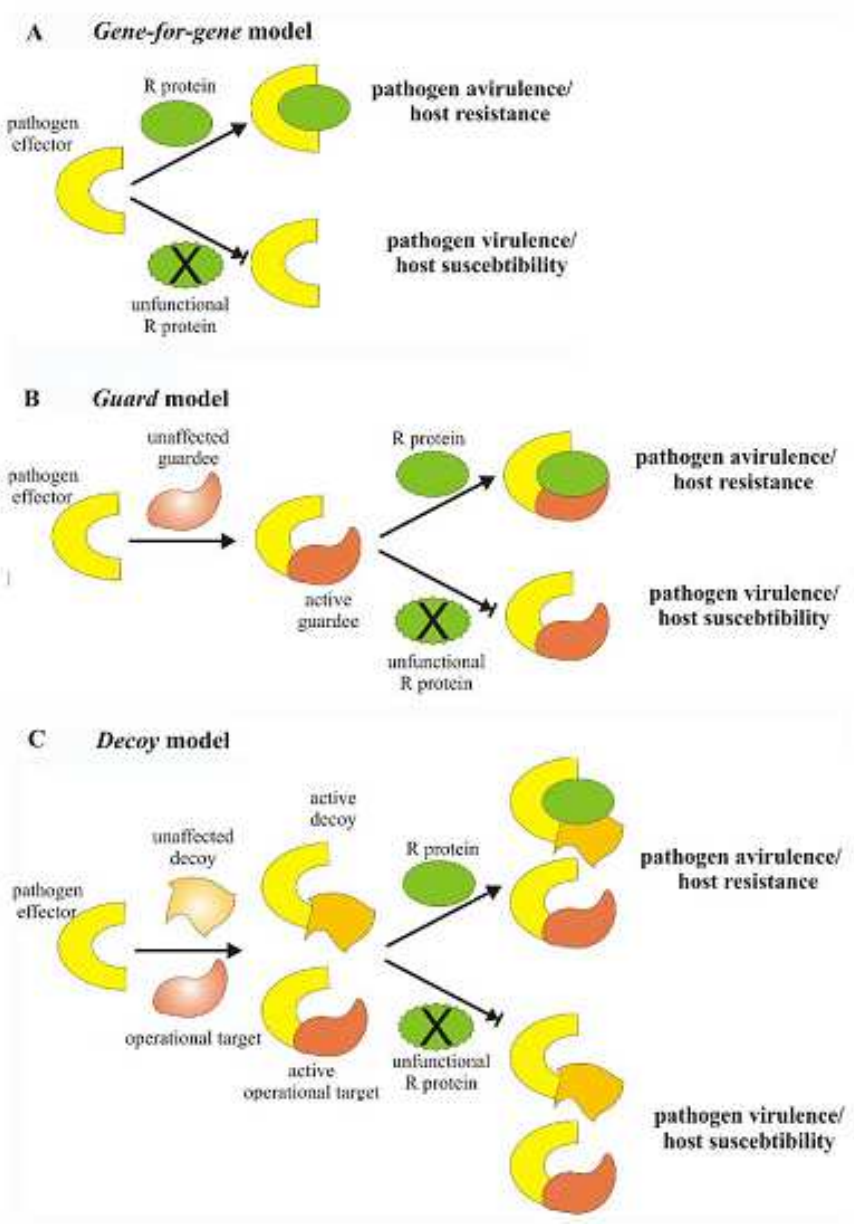
O modelo *decoy* de interação é uma segunda variação da hipótese gene-a-gene. No modelo decoy, proteínas especificas, que são similares àquelas proteínas alvos dos efetores patogênicos (proteínas iscas), são produzidas pela planta durante a interação planta-patógeno. A função dessas proteínas *decoy* é se ligar aos efetores do patógeno e mediar as interações dos mesmos com as proteínas R (VAN DER HOORN; KAMOUN, 2008; GLOWACKI; MACIOSZEK; KONONOWICZ, 2010) (**Figura 6C**). Por exemplo, uma proteína efetora AvrPro de *Pseudomonas syringae* que infecta Arabidopsis, provoca uma resposta de resistência em plantas de tomateiro que possuem duas proteínas de resistência, Pto (*proteína decoy*, possui sítio Ser/Thr quinase) e interage com a Prf (NBS-LRR) sinaliza as respostas de defesa (MUCYN *et al*., 2007).

As proteínas R mais estudadas são receptores intracelulares chamados de NBS-LRR, contendo sítios de ligação a nucleotídeos (NBS), como ATP e GTP, e domínios ricos em leucina (LRR) (SOOSAAR et al., 2005). Ambos podem reconhecer proteínas efetoras, direta ou indiretamente, e ativar mecanismos de defesa que culminam com a PCD (do inglês, *Programmed Cell Death*), sendo, dessa forma, o tipo de resistência mediada por proteínas NBS-LRR, efetiva contra patógenos que apresentam um estilo de vida biotrófico, ou seja, necessitam de tecido vivo para se desenvolver, mas não contra patógenos necrotróficos, que se desenvolvem em tecido morto (COLL; EPPLE; DANGL, 2011; JONES; DANGL, 2006).

O domínio NBS é dividido em dois subdomínios ARC1 e ARC2 (do inglês, *Apoptosis R protein* and *CED-4* [*Cell Death Protein 4*]), que apresentam atividade de ATPase, estando envolvida com apoptose. O domínio LRR também possui dois subdomínios, que são importantes para o reconhecimento dos efetores. O primeiro grupo desses subdomínios, chamado de TIR (do inglês, *Toll/Interleukin Receptor-1 like domain*), é classificado por apresentar similaridade de sequência com o receptor de interleucina 1 de células animais, enquanto que o segundo grupo é representado pelo tipo CC (do inglês, *Coiled Coil like domain*), que possui uma hélice super enrolada (WHITHAM *et al*., 1994; MOFFETT, 2009; RONDE; BUTTERBACH; KORMELINK, 2014).

Após reconhecimento das moléculas efetoras do patógeno, várias respostas bioquímicas são induzidas, envolvendo transdução de sinais, resultando em respostas locais e sistêmicas. Essas respostas iniciais são baseadas na explosão oxidativa, com a liberação de ROS e RNS, resultando na HR (do inglês, *Hypersensitve Response*), estando relacionada com a PCD e indução da expressão de genes de defesa (PITZSCHKE; FORZANI; HIRT, 2006; COLL; EPPLE; DANGL, 2011; MOLASSIOTIS; FOTOPOULOS, 2011).

Figura 6. Modelos de reconhecimento planta-patógeno. (A) Modelo de reconhecimento gene-a-gene, um reconhecimento específico direto do efetor (proteína Avr) do patógeno pelo receptor do hospedeiro (proteína R) dando início as respostas de defesa. (B) Modelo de reconhecimento "guarda", em que uma proteína guarda se liga ao efetor do patógeno auxilia o reconhecimento para que a resposta de resistência seja iniciada. (C) Modelo de reconhecimento "*decoy".* Uma proteína mimetiza uma proteína alvo do efetor patogênico, e após o reconhecimento, interage com uma proteína R disparando as repostas de defesa.



Fonte: GLOWACKI; MACIOSZEK; KONONOWICZ, 2010.

Após ocorrer HR, a SAR (do inglês, *Systemic Acquired Resistance*) é desencadeada, tendo como principal sinalizador o ácido salicílico (SONG; CHOI; RYU, 2013). A SAR é essencial na defesa da planta por 4 motivos importantes: 1) desenvolvimento de resistência em locais distantes do sítio da infecção, ou seja, em outros tecidos que não estão infectados (LUNA *et al.,* 2011); 2) atividade contra amplo espectro de patógenos, incluindo vírus, fungos, bactérias e nematoides, além daquele que desencadeou a SAR (SONG; RYU, 2013); 3) proteção de longa duração (semanas, meses ou, até mesmo, uma estação inteira), também conhecida como "memória vegetal"; e 4) expressão de genes relacionados à síntese de PR-proteínas (do inglês, *Pathogenesis-Related Proteins*) (BOATWRIGHT; MUKHTAR, 2013).

1.4 Interação Planta-Vírus

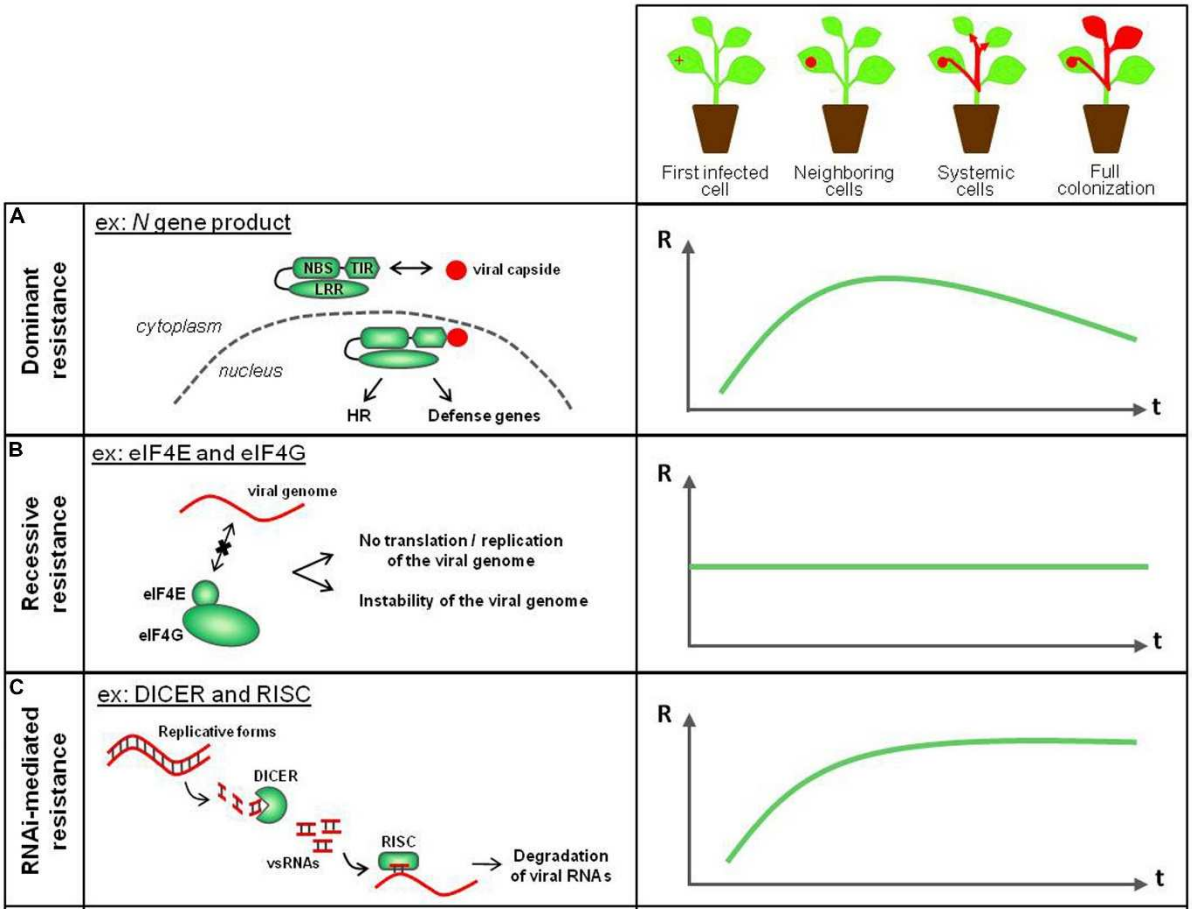
A interação planta-vírus apresenta algumas características únicas quando comparadas com outras interações planta-patógeno. Hull (2009) discute a existência de diversas relações possíveis no patossistema planta-vírus (**Tabela 1)**. Somado a isso, Nicaise (2014) discute que a resistência de plantas contra vírus pode envolver vários mecanismos como resistência dominante, recessiva e mediada por RNAi, sendo estas, etapas complementares da resistência, que são divididos em escala temporal (no início ou nos passos da infecção tardia) e espacial (no sítio de infecção ou em tecidos sistêmicos) sempre tendo como alvo e ter as moléculas derivadas de vírus como alvo (**Figura 7**) (NICAISE, 2014).

Tabela 1 – Tipos de Interações planta-vírus envolvidos na resistência e susceptibilidade à infecção

Resistência Recessiva (Planta não hospedeira)	Imunidade total	O vírus não se replica devido à sua incapacidade de reconhecer fatores da planta, essenciais para sua replicação**.**
Resistência Dominante (Planta hospedeira)	Altamente resistente	A multiplicação viral é limitada às células inicialmente infectadas. A planta consegue conter a doença devido à ativação das respostas de defesa, que impedem o estabelecimento da doença.
	Resistente	A infecção é limitada a uma zona de células em volta do sítio de tentativa de infecção, havendo formação de lesões necróticas locais.
	Susceptível	Replicação do vírus e estabelecimento dos sintomas severos da doença.

Fonte: Hull (2009).

FIGURA 7. Mecanismos de defesa antiviral em plantas. (A) Resistência dominante mediada por proteínas NBS-LRR (gene R), por meio do reconhecimento de proteínas Avr (gene Avr) dos patógenos. Essa resposta está associada com HR, que restringe o sítio de infecção viral. (B) Resistência recessiva, corresponde à ausência de fatores apropriados das plantas, que são requeridos para o ciclo de infecção viral. (C) Resistência por RNAi, que utiliza como alvo os ácidos nucleicos derivados dos vírus. Uma vez ativado, esse mecanismo de defesa aumenta e se espalha para toda a planta, tendo duração de alguns dias.



Fonte: Nicaise (2014)

1.4.1 Resistência Dominante

A resistência dominante é uma forma de defesa das plantas contra vírus que é promovida pelas proteínas NBS-LRR, codificadas pelos genes (*R*) de resistência dominante, que atuam reconhecendo os produtos dos genes Avr de avirulência, iniciando mecanismos de defesa ETI e evitando a infecção viral (**Figura 7A)** (MARTIN; BOGDANOVE; SESSA, 2003; ROBAGLIA; CARANTA, 2006; RONDE; BUTTERBACH; KORMELINK, 2014). Diversas proteínas NBS-LRR têm sido descritas desempenhando papel crucial na defesa contra a infecção viral. Essas respostas podem ser mediadas por TIR-NBS-LRR ou CC-NBS-LRR, pelo reconhecimento direto ou indireto, dos efetores virais, ou através da interação com proteínas (*guardee*) que sofreram modificações induzidas por efetores virais.

As respostas de defesa mediadas por proteínas receptoras tipo TIR, são bem descritas em plantas de *Nicotiana tabacum*, que apresentam um gene de resistência dominante R, codificante para uma proteína do tipo TIR-NBS-LRR, que interage com um domínio helicase de uma replicase do TMV (*Tobacco Mosaic Virus,* Gênero - *Tobamovirus*), desencadeando respostas de defesa (UEDA; YAMAGUCHI; SANO, 2006). Já em plantas de *Solanum tuberosum*, o gene Rx1 codifica uma proteína tipo CC-NB-LRR, que atua reconhecendo a proteína do capsídeo viral do PVX (*Potex Virus X*, Gênero *Potexvirus*), induzindo as respostas de defesa da planta (RAIRDAN *et al*., 2008).

Tanto em *N*. *tabacum* quanto em *S. tuberosum*, a ativação das NBS-LRR inicia uma cascata de sinalização celular mediada por proteínas MAPK envolvendo aumento do influxo de Ca^{2+}, induzindo HR e consequente PCD, mediada pelo complexo EDS1/PAD4/SAG101 (do inglês, *Enhanced Disease Susceptibility 1* [EDS1] / *Phytoalexin Deficient 4* [PAD4] / *Senescence-Associated Gene 101* [SAG101]), com o intuito de impedir que o vírus (organismo biotrófico) estabeleça infecção sistêmica (AZEVEDO *et al*., 2006; PALUKAITIS; CARR, 2008; COLL; EPPLE; DANGL, 2011; ZHU *et al*., 2011; KUNDU *et al.,* 2013; NICASE, 2014).

1.4.2 Resistência Recessiva

A resistência recessiva (imunidade) caracteriza-se pela incapacidade do vírus em infectar o hospedeiro, devido à perda de compatibilidade entre proteínas codificadas pelo vírus com as proteínas do hospedeiro, processo necessário para replicação viral e estabelecimento da infecção (ROBAGLIA; CARANTA, 2006; MOFFETT, 2009) (**Figura 7B**). Diversos genes envolvidos na resistência recessiva em plantas foram identificados, clonados e caracterizados em várias culturas vegetais, sendo todos codificantes para proteínas pertencentes as famílias dos fatores de iniciação de tradução (eIF) os quais são proteínas necessárias para replicação viral no hospedeiro, indicando que esse tipo de resistência é mais comum contra vírus (TRUNIGER; ARANDA, 2009; NICASE, 2014).

Este tipo de resistência ocorre, preferencialmente, em protoplastos de folhas inoculadas, local onde ocorre o processo de replicação viral, influenciando a multiplicação do vírus na célula, bem como o movimento célula a célula. Os genes envolvidos na resistência recessiva mais estudados são os que codificam para fatores de iniciação da tradução das familias 4E (eIF4E) e 4G (eIF4G) que juntos formam o complexo eIF4F, e suas respectivas isoformas, eIF(iso)4E e eIF(iso)4G, formando o eIF(iso)4F, tendo com principal função a ligação ao capacete do RNAm maduro (7-metilguanosina) da planta, essencial no processo de tradução (GALLIE; BROWNING, 2001).

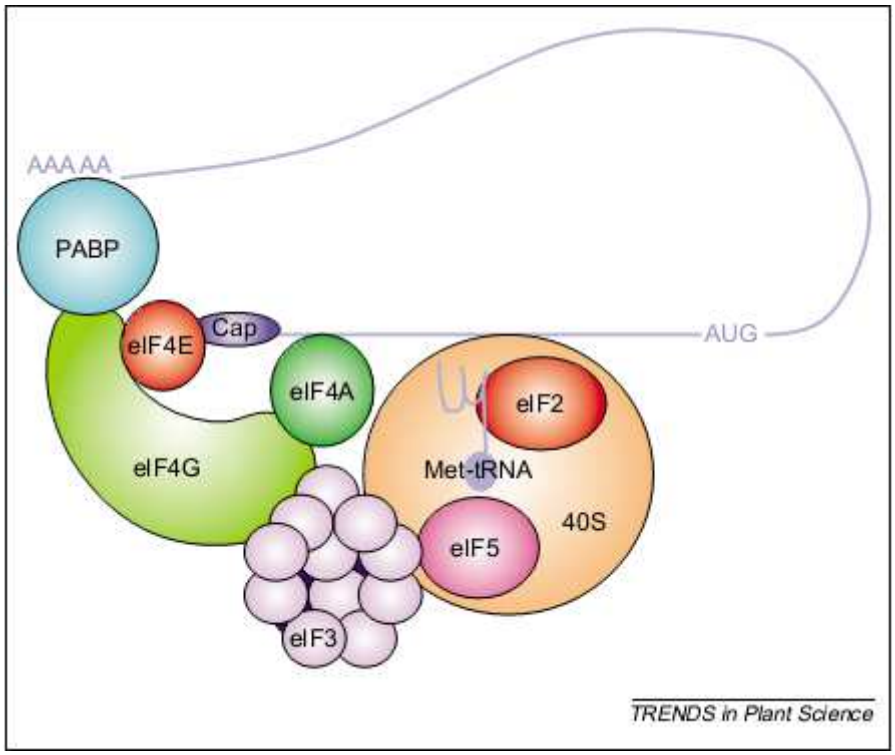
A subunidade eIF4E é uma proteína de 24 kDa, que possui o sítio de ligação ao capacete do RNAm maduro (7-metilguanosina) da planta (GOODFELLOW; ROBERTS, 2007). Já a subunidade eIF4G é uma proteína de ~200 kDa que interage com eIF4E e outros fatores de iniciação, incluindo eIF4A, eIF3 e com uma proteína de ligação à cadeia poliadelinada (**Figura 8**) (BROWNING, 2004; ROBAGLIA; CARANTA, 2006).

Estudos com vírus do gênero *Potyvirus* mostraram que a resistência de plantas pode estar associada com a incapacidade da VPg em se ligar ao eIF4E ou eIF(iso)4E das plantas (GRZELA *et al*., 2006). Essa incapacidade de ligação é o resultado de mutações em aminoácidos expostos na superfície dos fatores de iniciação da tradução das familias 4E, podendo ser no domínio de ligação ao capacete ou não,

que impede o reconhecimento do eIF4E ou eIF(iso)4E pela VPg, resultando na inibição da multiplicação do vírus (YEAM *et al*., 2007).

Nicaise et al. (2003) demonstraram que a substituição de apenas um aminoácido (alanina para prolina) na sequência do eIF4E foi suficiente para conferir resistência em alface contra o LVM (*Lettuce Mosaic Vírus*, gênero *Potyvirus*). Outros componentes pertencentes à maquinaria de síntese de proteínas são recrutados pelo vírus para favorecer o processo de replicação. Dentre esses, se desacatam os fatores de elongação eEF1A e eEF1B e as proteínas ligantes à cauda poli-A (PABs) 2, 4 e 8 (SASVARI *et al*., 2011; HWANG *et al*., 2013 e 2015). O conhecimento da função desses fatores de tradução no mecanismo de resistência pode conduzir ao desenvolvimento de novas estratégias de controle de doenças virais.

FIGURA 8. Complexo de iniciação da tradução de proteínas em plantas. O RNAm ligado ao capacete 5´ é recrutado pelo complexo eIF4F, que é composto por duas subunidades, eIF4E e a eIF4G.



Fonte: ROBAGLIA; CARANTA, 2006.

1.4.3 Silenciamento por RNA de interferência (RNAi)

Recentemente, foi descoberto um novo mecanismo de defesa induzido, que está presente em plantas, e em quase todos os reinos, chamado de silenciamento por RNA de interferência (RNAi). O RNAi é comandado por molécula de sRNAs (do inglês, *Small RNAs*) que são pequenas moléculas de RNA não codificantes, com tamanho que varia entre 20-30 nucleotídeos. RNAi é um mecanismo de inibição específica da expressão gênica, tendo como alvos os ácidos nucleicos virais (**Figura 7C**), que está envolvido na regulação de diversos processos fisiológicos das plantas, sendo a principal via de defesa de plantas contra vírus (PUMPLIN; VOINNET, 2013; SHUKLA; DALAL; MALATHI, 2013).

A classificação dos sRNAs em plantas é dividida em dois grupos, de acordo com o seu precursor e via de biogênese: miRNA (do inglês, *microRNA*) e siRNA (do inglês, *small interfering RNA*). Os miRNAs possuem de 21-24 nucleotídeos e são originados a partir de moléculas de RNA com emparelhamento de bases imperfeito, que formam estruturas em grampo. Os siRNA possuem de 23-30 nucleotídeos e são gerados a partir de dsRNA (do inglês, *double-stranded RNAs*), e requerem a atividade da enzima RNA polimerase RNA-dependente (BARTEL, 2009; KATIYAR-AGARWAL; JIN, 2010).

Os sRNAs controlam a expressão gênica em plantas ou patógenos por dois mecanismos distintos, o silenciamento gênico pós-transcricional (PTGS, do inglês, *Post-Transcriptional Gene Silencing*) ou silenciamento gênico transcricional (TGS, do inglês, *Transcriptional Gene Silencing*). Tanto miRNA e siRNA podem induzir PTGS por meio da clivagem/degradação ou inibição da tradução pelo complexo denominado de RISC (do inglês, *RNA-Induced Silencing Complex*) (JOVEL; WALKER; SANFAÇON, 2011; SABIN; CHERRY, 2013), que é constituído por diversas ribonucleoproteínas, Dicer, proteína TRBP (do inglês, three double-stranded RNA-binding domains protein), proteína Argonauta (função de RNase) representa o centro do complexo, e siRNA ou miRNA. Após reconhecimento do dsRNA (geralmente de vírus), as enzimas Dicers atuam clivando o dsRNA em pequenos fragmentos duplexes que são incorporados no complexo citoplasmático RISC. Por outro lado, TGS atua na regulação gênica por meio da metilação do DNA, modificação das histonas ou, até mesmo, modificação na cromatina, que, usualmente, é realizado por

siRNA, muito embora algumas classes específicas de miRNAs possam realizar esse processo (INCARBONE; DUNOYER, 2013; PUMPLIN; VOINNET, 2013).

Diversos trabalhos citam a importância do silenciamento por RNAi na defesa de plantas contra vírus. Recentemente, Garcia-Ruiz e colaboradores (2010) mostraram que o silenciamento por RNAi é essencial para a defesa de plantas de *Arabidopsis* frente ao TNV (do inglês, *Turnip Mosaic Virus*, gênero *Potyvirus*). Cruz e Aragão (2013) demonstraram que plantas transgênicas de feijão-de-corda, habilitadas a inibir a produção do cofator da protease viral via RNAi, apresentaram resistência ao CPSMV quando comparadas com as plantas não transformadas, suscetíveis ao vírus. Esses resultados demonstram a importância do silenciamento por RNAi nas respostas de defesa do feijão-de-corda ao ataque do CPSMV.

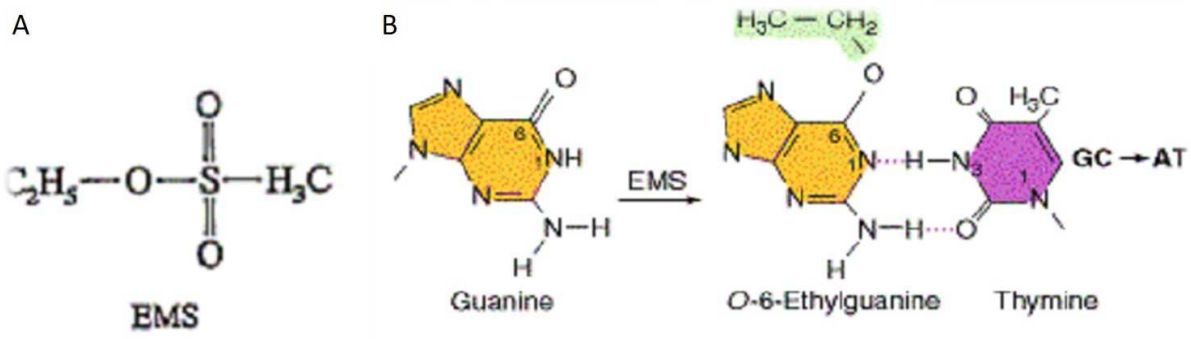
1.5 Etil Metanosulfonato (EMS)

O Etil metanosulfonato (EMS) é um agente mutagênico químico ($C_3H_8SO_3$, **Figura 9A**), que induz mutações aleatórias, por substituição de um único nucleótideo no material genético. O EMS causa mutação por interagir, preferencialmente, com resíduos de guanina transformando-os em O-*6*-etilguanina (**Figura 9B**), uma base anormal, que é adicionada naturalmente nas moléculas de DNA pela DNA polimerase (LOVELESS, 1958). Entretanto, durante a replicação do DNA mutado, a timina, em vez de citosina, é quem é adicionada na nova fita que está sendo sintetizada, para emparelhar com O-*6*-etilguanina. Essa alteração resulta na substituição de pares G≡C por A=T, durante o processo de replicação, aumentando o conteúdo A=T em relação ao G≡C, na molécula de DNA nascente. Essa mutação se acumula durante o processo de replicação do DNA e, dessa forma, é transmitidas para a novas células (COOPER *et al*., 2008; GREENE *et al*., 2003).

Vários estudos mostram a utilização do EMS como agente mutagênico em diversas culturas, como soja (KHAN; TYAGI, 2013), tomate (SHIRASAWA *et al*., 2015) e melão (GALPAZ *et al*., 2013), com diversas finalidade de estudos. Em alguns desses trabalhos plantas mutagenizedes produzias a partir do tratamento de sementes com EMS foram utilizadas para tentar entender a interação planta-vírus em

Arabdopsis thaliana (LELLIS *et al*., 2002) e tomate (*Solanum lycopersicum*) (PIRON *et al*., 2010). Lellis *et al*. (2002), por exemplo, demonstraram que o tratamento de sementes de *A. thaliana* susceptíveis ao TuMV (do inglês, *Turnip Mosaic Virus*, Gênero *Potyvirus*) com EMS (0,002%) produziu plantas que apresentaram o fenótipo de resistência quando desafiadas com o vírus, sendo esse fato denominado, pelos autores, como “perda de susceptibilidade”. Nesse caso, o EMS induziu mutações nas sequências eIF(iso)4E, que resultou na mudança conformacional desse fator e impediu a ligação da VPg, inibindo a replicação do vírus.

Figura 9. (A) estrutura química (B) mecanismo de ação do EMS.



Fonte: http://pharmacology.unmc.edu/cancer/antibio.htm

2. JUSTIFICATIVA

O feijão-de-corda apresenta importante papel socioeconômico no mundo e no Brasil, principalmente nas regiões Norte e Nordeste, sendo o principal alimento das famílias residentes nessas regiões (BESERRA-Jr *et al.,* 2011). Entretanto, mesmo tendo grande importância na alimentação, sua produção é muito baixa, devido, dentre muitos fatores, às doenças causadas por vírus, fungos e nematoides, destacando-se os primeiros (NEVES *et al.,* 2011). As doenças causadas por vírus estão entre os grandes responsáveis pela baixa produção do feijão-de-corda (LIMA; SITTOLIN; LIMA, 2005) e, no Brasil, a principal delas é aquela causada pelo vírus do mosaico severo do caupí (CPSMV) (LIMA *et al.,* 2012).

É relatada a existência de diversos cultivares ou genótipos de feijão-de-corda que são resistentes ou susceptíveis ao CPSMV. Contudo, poucos estudos foram conduzidos na busca de fatores ou características que definem a resistência ou a susceptibilidade do feijão-de-corda ao vírus CPSMV. Estes trabalhos atribuem a herança recessiva como principal fator que tornam alguns cultivares resistentes ao CPSMV. Mas, nenhum deles explica os mecanismos de defesa envolvidos, vias bioquímicas, proteínas que estão relacionadas à defesa ou, até mesmo, compostos secundários como compostos fenólicos, que possam estar relacionados ao estado de resistência.

Atualmente, o método mais eficaz e prático de prevenir as perdas na produção ocasionadas por infecções pelo CPSMV é a utilização de cultivares imunes ao vírus CPSMV, como CNC-0434 e Macaibo, provenientes de cruzamentos entre diversos genótipos, ou cultivares resistentes. Entretanto, recentemente, foi descoberto um isolado do CPSMV que é capaz de infectar o cultivar Macaíbo, sendo esse vírus, devido a esse fato, denominado de CPSMV-MC. Nesse contexto, a utilização de cultivares imunes pode não ser uma alternativa viável para o controle do CPSMV, ressaltando a necessidade de se entender os genes e vias bioquímicas envolvidos na imunidade e/ou resistência ao CPSMV.

Portanto, visto a escassez de trabalhos relacionados ao patossistema feijão-de-corda x CPSMV, com enfoque nos mecanismos de defesa do feijão-de-corda, no

presente trabalho foi realizada análise comparativa de parâmetros fisiológicos, bioquímicos e moleculares entre um genótipo de feijão-de-corda suscetível (CE-31 sin. Pitiúba) e seu derivado mutagenizede resistente, induzido por EMS, ambos desafiados com o CPSMV. Nesse contexto, essa proposta foi dividida em dois subprojetos, a saber : (1) Avaliação quantitativa de algumas propriedades bioquímicas e fisiológicas e (2) Análise proteômica. Tais abordagens foram escolhidas, visando entender que fatores bioquímicos e/ou fisiológicos estão associados aos fenótipos de suscetibilidade e resistência do feijão-de-corda ao CPSMV, sendo passos importantes que auxiliarão com informações valiosas para o futuro desenvolvimento de plantas transgênicas resistentes ao CPSMV.

3. HIPÓTESE

O atual conhecimento sobre os mecanismos utilizados pelas plantas para se defender contra patógenos agressores demonstra um sofisticado e complexo mecanismo de defesa que consiste em várias linhas de defesa, que atuam suprimindo ou ativando fatores de resistência nas plantas contra os vírus. Diversos estudos têm mostrado que o tratamento de sementes susceptíveis à infecção viral com EMS pode resultar em plantas mutagenizedes que apresentam fenótipo de resistência frente à infecção causado por vírus.

Baseado nessas observações, as seguintes hipóteses foram formuladas:

1) A mutação para resistência ao vírus induzida pelo tratamento das sementes com EMS altera genes relacionados com vias bioquímicas e fisiológicas de defesa da planta mutagenizede resistente, conferindo-lhe a capacidade de reconhecer a infecção viral e induzir mecanismos de defesa (acúmulo de ROS, PCD e enzimas relacionadas à defesa) para contra-atacar a infecção viral, a fim de evitar o estabelecimento da doença;

2) Como genes das plantas mutagenizedes resistentes foram alterados pelo tratamento com EMS, então, houve reprogramação genética e consequente alteração no perfil proteômico dessas plantas, resultando no acúmulo de proteínas importantes para defesa da planta contra o vírus.

Em decorrência dessas hipóteses, as seguintes perguntas foram elaboradas como guias para definição da abordagem experimental proposta nesse projeto;

1. Sementes de feijão-de-corda susceptível ao CPSMV quando tratadas com EMS geram plantas mutagenizedes resistentes ao vírus?
2. As plantas de feijão-de-corda mutagenizedes, resistentes, apresentam alteração na cinética de enzimas relacionadas com a defesa vegetal?
3. Existe reprogramação genética que leva ao aumento ou diminuição no acúmulo de proteínas nas plantas de feijão-de-corda mutagenizedes em

relação às plantas controles? Em caso positivo, essa alteração no conteúdo proteico se correlaciona com a resistência ao CPSMV, em plantas de feijão-de-corda mutagenizedes?

4. Plantas de feijão-de-corda mutagenizedes apresentam acúmulo de H_2O_2 e compostos do metabolismo secundário da planta (compostos fenólicos e lignina) em relação às plantas controles?

5. Existe alteração nos parâmetros fisiológicos (fotossíntese líquida, condutância estomática e conteúdo de clorofila) nas plantas mutagenizedes em relação às plantas controles? Se sim, essa alteração está envolvida com a resistência?

4. OBJETIVOS

4.1 Objetivo geral

- Investigar as respostas de defesa bioquímicas e fisiológicas das plantas de feijão-de-corda mutagenizedes e não mutagenizedes, ambas infectadas com o CPSMV.

4.2 Objetivos específicos

- Detectar a presença do CPSMV nas folhas de plantas de feijão-de-corda mutagenizedes e não mutagenizedes, ambas infectadas com o CPSMV.

- Avaliar a cinética enzimática de algumas PR-proteínas [Quitinase, β-1,3-glucanase e Peroxidase de fenóis (POX)], bem como enzimas envolvidas no metabolismo das espécies reativas de oxigênio [Superóxido dismutase (SOD), catalase (CAT), peroxidase do ascorbato (APX)] e a enzima responsável pela via de produção dos compostos fenólicos, fenilalanina amônia liase (PAL), nas folhas de plantas de feijão-de-corda mutagenizedes e não mutagenizedes, ambas infectadas com o CPSMV

- Investigar o acúmulo de peróxido de hidrogênio (H_2O_2), compostos fenólicos e lignina, nas folhas de plantas de feijão-de-corda mutagenizedes e não mutagenizedes, ambas infectadas com o CPSMV.

- Avaliar a presença de morte celular programada (PCD) nas folhas de plantas de feijão-de-corda mutagenizedes e não mutagenizedes, ambas infectadas com o CPSMV.

- Avaliar os parâmetros fisiológicos relacionados à fotossíntese, conteúdo de clorofila e carotenoides.

- Realizar análise proteômica comparativa entre as proteínas de folhas de plantas de feijão-de-corda mutagenizedes e não mutagenizedes, usando LC-ESI-MS/MS.

- Analisar os perfis espectrométricos obtidos de folhas de plantas de feijão-de-corda mutagenizedes e não mutagenizedes, usando o programa *Data Analysis*.

- Identificar, classificar e categorizar as proteínas diferencialmente acumuladas em folhas de feijão-de-corda mutagenizedes e não-mutagenizedes, ambas expostas ao CPSMV.

- Identificar as alterações celulares (vias bioquímicas e organelas subcelulares) envolvidas no processo resistência (planta mutagenizedes resistentes) e susceptibilidade (planta controles).

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CAPÍTULO II

Photosynthetic and Biochemical Mechanisms of an EMS-Mutagenized Cowpea Associated with its Resistance to *Cowpea Severe Mosaic Virus*

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Photosynthetic and Biochemical Mechanisms of an EMS-Mutagenized Cowpea Associated with its Resistance to *Cowpea Severe Mosaic Virus*

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Abstract

Cowpea is an important crop that makes major nutritional contributions particularly to the diet of the poor population worldwide. However, its production is low because cowpea is naturally subjected to several abiotic and biotic stresses, including viral agents. *Cowpea severe mosaic virus* (CPSMV) drastically affects cowpea grain production. This study was conducted to compare photosynthetic and biochemical parameters of a CPSMV-susceptible cowpea (CE-31 genotype) and its derived ethyl methanesulfonate (EMS)-mutagenized resistant plantlets, both challenged with the virus, to shed light on the mechanisms of resistance to CPSMV. Virus inoculation was done in the fully expanded trifoliate secondary leaf, 15 days after planting. At 7 DPI, *in vivo* photosynthetic parameters were measured and leaves collected for biochemical analysis. CPSMV-inoculated mutagenized-resistant cowpea plantlets (MCPI) maintained high photosynthesis index and increased chlorophyll and carotenoid contents in relation to CPSMV-susceptible inoculated cowpea (CPI). Visually, MCPI did not exhibit disease symptoms, supported by the absence of virus particles infecting the cowpea leaves as judged by RT-PCR, apparently associated with reduction in CAT and APX and increase in SOD and GPOX activities, which led to increased H_2O_2 and phenolic contents and cell wall lignifications**.** Moreover, chitinase and phenylalanine ammonia lyase activities were also increased in MCPI plantlets. All together these photosynthetic and biochemical changes might have contributed for the resistance of MCPI. Contrarily, CPI plantlets showed CPSMV accumulation, severe disease symptoms, reduction in the photosynthesis-related parameters, and decreased chlorophyll, carotenoid, phenolic compound, and H_2O_2 contents, in addition to increased *β*-1, 3-glucanase, and catalase activities that might have favored viral infection.

Key words: Cowpea, EMS, CPSMV, plant defense, photosynthesis, antioxidant enzymes, PR-proteins

Abbreviations - ΔF/Fm′, actual PSII quantum efficiency; Ci, internal pressure of CO_2; CO_2, carbon dioxide; CPSMV, Cowpea Severe Mosaic Virus; EMS, Ethyl methanesulfonate; Fo, minimal fluorescence from dark-adapted leaf; Fo', minimal fluorescence from light-adapted leaf; Fm, maximal fluorescence from dark-adapted leaf; Fm', maximal fluorescence from light-adapted leaf; Fs, steady state fluorescence; Fv/Fm, maximum PSII quantum efficiency; gs, stomatal conductance; HR, hypersensitive response; H_2O_2, hydrogen peroxide; NBT, nitroblue-tetrazolium; NPQ, non-photochemical quenching; PPFD, photosynthetic photon flux density; PCD, programmed cell death; P_N, net CO_2 assimilation rate; PR-proteins, pathogenesis-related proteins; qP, photochemical quenching; ROS, reactive oxygen species; RT-PCR, reverse transcription-polimerase chain reaction.

1. Introduction

Cowpea is an important crop that makes major nutritional contributions as a source of proteins and carbohydrates particularly to the diet of poor people worldwide. However, its production is impaired due to various stresses, including those caused by viruses. *Cowpea severe mosaic virus* (CPSMV) belongs to the *Comovirus* genera and constitutes the main virus that attacks cowpea because it causes important losses in the crop production and yield (Booker et al. 2005). In general, virus-infected plants show severe chlorosis symptoms and leaf deformation, which affect the normal plant growth and development hindering its productivity (Laliberté and Zheng 2014, Rys et al. 2014).

Photosynthesis is one the most important metabolic processes in plants that is affected by viral infection in compatible interactions. Under virus infection, susceptible plants present reduction in chlorophyll content, lower activity of light-harvesting complex (LHC), and reduction in photosynthesis-related processes (non-photochemical quenching, effective quantum yield at photosystem II, and CO_2 assimilation) (Liu et al. 2014, Rys et al. 2014, Shimura et al. 2011). Besides generating energy as ATP and reduction power in the form of NADPH for biosynthetic processes, photosynthesis has a role in plant defense response to pathogen attack. Indeed, during photosynthesis, chloroplasts produce reactive oxygen species (ROS), especially H_2O_2, which have fundamental role in the plant defense mechanisms both directly by acting as antimicrobial/antiviral agent and indirectly by inducing hypersensitive response (HR), programmed cell death (PCD), strengthening of the plant cell walls via cross-linking of glycoproteins, and induction of defense related genes (Jones and Dangl 2006, Torres 2010). Amongst the genes induced, are those that encode the pathogen-related proteins (PR-proteins). PR-proteins are classified into 17 families and are all associated with the plant resistance to pathogen infection (Soosaar et al. 2005, Van Loon et al. 2006).

Thus, in an incompatible interaction, plants first recognize viral elicitors and/or effectors that activate the biochemical and physiological defense mechanisms towards preventing local infection, cell-to-cell movement, and systemic infection. At the earlier stage of attempted infection, a rapid production and accumulation of ROS occur in chloroplasts and mitochondria leading to effective defense responses (HR followed by

PCD), which limits infection, to individual cells and thus virus spreading to health tissues (Bolton 2009, Kundu et al. 2013).

Ethyl methanesulfonate (EMS, $C_3H_8SO_3$) is a chemical mutagen that induces random point mutations in guanine, producing abnormal *O*-6-ethylguanine that is added naturally in DNA, resulting in substitution of the original G:C base pair by A:T pair (transition mutation) after successive replication steps (Loveless 1969). In many cases, mutation caused by EMS in plants results in resistance to viruses (Lellis et al. 2002, Piron et al. 2013).). For example, EMS-treated *Arabidopsis thaliana* seeds susceptible to TuMV (*Turnip Mosaic Virus,* genus *Potyviruse*s) produced plants that showed resistance against TuMV. This phenomenon has been termed as "*loss of susceptibility*" (Lellis et al. 2002).

In the present study, we compared photosynthetic and biochemical parameters of a CPSMV-susceptible cowpea (CE-31 genotype) and its derived EMS-mutagenized resistant plantlets, both challenged with the virus, to shed light on the mechanisms of resistance to CPSMV.

2. Materials and Methods

2.1 Plant material and treatment of cowpea seeds with EMS

Cowpea seeds from the CE-31 genotype (sin. Pitiuba) were obtained by the Laboratory of Plant Defense Proteins (LPVD), Department of Biochemistry and Molecular Biology, Federal University of Ceara, Brazil, from successive cultivations in an open field. Mature seeds were disinfected with 1% (v/v) sodium hypochlorite (0.05% active chlorine) for 3 min, exhaustively washed (10x), and incubated for 20 min in sterile ultra-pure water. After blotted dried, seeds were immersed in 0.04% (v/v) EMS (Lellis et al. 2002) for 20 h, as established in pilot experiments, and washed exhaustively (30x) with sterile ultra-pure water (300 mL each wash) to remove excess EMS.

2.2 Growth conditions

Mature cowpea seeds, from the CE-31 genotype, highly susceptible to *Cowpea Severe Mosaic Virus* ($CPSMV_{CE}$ isolate from Ceara state, Brazil, thereafter referred simply as CPSMV), were surface-disinfected as above and incubated for 20 min with sterile ultra-pure water, at room temperature (23 ± 2 °C) to break dormancy. Seed germination was done in filter papers (Germitest®, 28 x 38 cm) watered twice a day with sterile ultra-pure water. Three days later, germinated seeds were selected and transplanted to 1.5 L pots (three per pots), containing river sand previously washed with tap water (5x) followed by distilled water (3x) 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 µmol m^{-2} s^{-1} (190SA quantum sensor, LI-COR, USA), around 12 h photoperiod, temperatures of 27.0 ± 0.8 °C (night) and 31.0 ± 3.0 °C (day), and 79.8 ± 10.9 % relative humidity. The cowpea plantlets were watered once daily with sterile distilled water (100 mL per pot) for up to 3 days after they were transplanted to the pots and next with a nutritive solution (Hoagland and Arnon 1959) modified by (Silveira et al. 2001).

2.3 Virus inoculum preparation and cowpea plantlet inoculation

The CPSMV inoculum was obtained from the virus infected CE-31 genotype leaves showing typical symptoms of severe mosaic. Plantlets 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. Infected leaves were macerated (1:10, m/v) with 10 mM K^+-phosphate buffer, pH 7.0, containing 0.01% (m/v) sodium sulfite and the resulting suspension mixed with 500-600 mesh carborundum powder (1:10, m/v), used as abrasive (Paz et al. 1999). Three groups of cowpea plantlets (Table 1) were used in the experiments: CPU group, plantlets grown from the non-mutagenized CE-31 seeds that were mock inoculated (virus free buffer + carborundum); CPI group, plantlets grown from non-mutagenized CE-31 seeds, but challenged with CPSMV; and MCPI group,

plantlets grown from the CE-31 seeds that were previously treated with 0.04% (v/v) EMS and also challenged with CPSM. MCPI represented mutagenized plantlets that became resistant to CPSMV isolate. Virus inoculation was done 15 days after the plantlets were transplanted to pots, when they were at the three-leaf growth stage, by gentle rubbing the CPSMV suspension on the adaxial and abaxial surfaces of the secondary leaves positioned individually between the tips of the index finger and thumb with hands protected with surgical gloves. The physiological measurements were done in the entire plantlets at seven days post inoculation (7 DPI) from 9:00 to 11:00 am. For the biochemical analyses, the leaves of CPU, CPI, and MCPI groups were detached at 7 DPI and stored at -80 °C until used. Quantification of hydrogen peroxide (H_2O_2) and phenolic compounds was done in fresh leaves collected at 7 DPI and immediately used for analysis. Plantlets were arranged in a completely randomized block design with 3 biological replicates per block (pot). The experiments were done with plantlets arranged in a completely randomized block design, repeated three times independently, with three biological replicates (pots) for each group, and every pot contained three cowpea plants. Results reported herein are represented by mean ± SD.

2.4 Gas exchange and chlorophyll a fluorescence

The physiological parameters were measured with a portable Infrared Gas Analyzer System (IRGA) (LI-6400XT, LI-COR, Lincoln, NE, USA), equipped with a leaf chamber fluorometer (LI-6400-40, LI-COR, Lincoln, NE, USA). Conditions inside the IRGA chamber during the measurements were photosynthetic photon flux density (PPFD) of 1000 μmol m^{-2} s^{-1}, 28 °C, 1.0 ± 0.2 kPa air vapor pressure deficit, and 0.038 kPa air CO_2 partial pressure. The amount of blue light was set to 10% of PPFD to maximize stomatal aperture (Flexas et al. 2008). The fluorescence parameters were measured using the method of light pulse saturation in both light- and 30 min dark-adapted leaves (Schreiber et al. 1994). The intensity and duration of the light saturation pulse were 8000 μmol m^{-2} s^{-1}, and 0.7 s, respectively. The photochemical parameters assessed were: maximum PSII quantum efficiency [Fv/Fm = (Fm − Fo)/Fm]; actual PSII quantum efficiency [ΔF/Fm′ = (Fm′ − Fs)/Fm′], in which 0.40 and 0.84 were used as the fraction of excitation energy distributed

to PSII, and the fraction of incoming light absorbed by leaves, respectively; photochemical quenching [qP = (Fm' – Fs)/(Fm' – Fo')]; and non-photochemical quenching coefficient [NPQ = (Fm − Fm′)/Fm′], in which Fm and Fo are the maximum and minimum fluorescence of dark-adapted leaves, and Fm′, Fo´, Fs are the maximum, minimum, and steady state fluorescence of the light-adapted leaves, respectively (Schreiber et al. 1994).

Table 1. Control (CPU) and experimental (CPI, MCPI) plant groups

Plant groups	0.04% EMS-treated	CPSMV-infected	CPSMV-free buffer	Disease symptoms
CPU[a]	No	No	Yes	No
CPI[b]	No	Yes	No	Yes
MCPI[c]	Yes	Yes	No	No

[a] Plants originated from cowpea (CE-31 genotype) seeds not previously treated with 0.04% (v/v) ethyl methanesulfonate (EMS), which were not inoculated with Cowpea severe mosaic virus (CPSMV).

[b] Plants originated from cowpea (CE-31 genotype) seeds not previously treated with 0.04% (v/v) EMS, but inoculated with CPSMV.

[c] Plants originated from cowpea (CE-31 genotype) seeds previously treated with 0.04% (v/v) EMS, and inoculated with CPSMV.

2.5 Chlorophyll and carotenoid contents

Total chlorophyll (Chl), chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid contents were determined spectrophotometrically as previously described (Lichtenthales and Welburn 1983). Frozen cowpea leaves (0.2 g) were immersed in 10 mL of 85% (v/v) aqueous acetone solution for 24 h in the dark and the suspension centrifuged at 4000 x *g*, 10 min, 4 °C. After suitable dilution, the absorbances of the supernatants were measured (Genesys 10S UV-Vis spectrophotometer, Thermo Scientific, USA) at 470 for carotenoids, and 646 and 664 nm for total chlorophyll, Chl *a*, and Chl *b*, respectively. Data are expressed in mg per gram of fresh weight (mg^{-1} g^{-1} FW).

2.6 Protein extraction and quantification

Frozen cowpea leaves (1 g) were taken from the ultrafreezer (-80 °C), pulverized to a fine powder with liquid nitrogen in a mortar and pestle, and the proteins extracted with 50 mM K^+-phosphate buffer pH 7.0, (1:4, m/v), for 10 min, under ice bath. The suspension was centrifuged at 13 000 *g* for 15 min, at 4 °C, and the supernatant dialyzed against the above buffer for 48 h (6 changes; 1:10 sample:buffer volume each), and centrifuged as above. All these steps were carried out at 4 °C. The resulting leaf extract was used for protein content measurement and enzyme assays all done in triplicate for every leaf extract sample. The leaf protein content was determined (Bradford 1976) using bovine serum albumin (BSA) as standard.

2.7 Superoxide dismutase (SOD; EC 1.15.1.1) activity

SOD activity was measured (Van Rossum et al. 1997) in the leaf extracts as the inhibition of the photochemical reduction of NBT (Nitroblue-tetrazolium; Sigma) to blue formazan, catalyzed by the enzyme. The leaf extract (0.05 mL) was added in microplate wells, in triplicate, containing 0.02 mL of 10^3 mM Na^+-phosphate buffer, pH 7.8, 0.01 mL 0.50% (v/v) Triton X-100, 0.02 mL 130 mM L-methionine, 0.01 mL 2 mM EDTA, 0.02 mL 0.75 mM NBT, 0.02 mL 1 mM riboflavin, and 0.05 mL of ultra-pure H_2O. Reaction took

place in a chamber under illumination by a 32-W fluorescent lamp and was terminated 5 min later by turning off illumination. The blue formazan generated by NBT photoreduction was measured as the increase in absorbance at 630 nm (Automated Microplate Reader, model ELX800-Bio-Tek Instruments®, Inc., USA). The control reaction mixture was prepared in the same manner as the experimental reaction, but without the leaf extract, and maintained in the dark. SOD activity was calculated as the difference between the absorbance of control and that of its experimental equivalent estimated for a 1-min reaction. One unit of SOD activity (1 UA) was defined as the amount of sample required to inhibit 50% of the NBT photoreduction (Beauchamp and Fridovich 1971). The enzyme activity was expressed in units of activity per gram of leaf fresh weight (UA g^{-1} FW).

2.8 Catalase (CAT; EC. 1.11.1.6) activity

Activity was assayed as previously described (Peixoto, 1999). The cowpea leaf extract (0.1 mL) and 12.5 mM H_2O_2 in 50 mM K^+-phosphate, pH 7.0, was incubated at 30 ºC for 10 min. Next 0.1 mL of the leaf extract was added to 0.90 mL of H_2O_2-phosphate buffer to start reaction. CAT activity was measured by the decrease in absorbance at 240 nm, in 30-second intervals up to 2 min (Havir and McHale 1987). CAT activity was expressed as µmol H_2O_2 oxidized per gram of leaf fresh weight per min (µmol H_2O_2 g^{-1} FW min^{-1}).

2.9 Ascorbate peroxidase (APX; EC. 1.11.1.11) activity

APX activity was determined according to Koshiba (1993) methodology at 30 ºC for 3 min. The reaction mixture was composed of the leaf extract (0.1 mL), 0.8 mL 50 mM K^+-phosphate buffer, pH 6.0, containing 0.5 mM ascorbate and 0.1 mL 2 mM H_2O_2. APX activity was measured by monitoring the decrease in absorbance at 290 nm for 3 min, as an index of ascorbate oxidation, and expressed as the concentration (µmol) of H_2O_2 per gram of leaf fresh weight per min (µmol H_2O_2 g^{-1} FW min^{-1}), taking into consideration that 2 mol ascorbate are required for reduction of 1 mol H_2O_2.

2.10 Guaiacol peroxidase (GPOX; EC 1.11.1.7) activity

GPOX activity was measured according to Urbanek et al. (1991), using guaiacol and H_2O_2 as substrates. Aliquots of 0.020 mL of the leaf extract were added to a solution composed of 0.5 mL 60 mM H_2O_2, 0.5 mL 20 mM guaiacol, and 0.980 mL 50 mM sodium acetate buffer, pH 5.2. Reaction was conducted at 30 °C and absorbances were taken at 480 nm in 10-second intervals up to 3 min. GPOX activity was expressed as concentration (µmol) of H_2O_2 per gram of leaf fresh weight per min (µmol H_2O_2 g^{-1} FW min^{-1}), taking into consideration that 4 mol H_2O_2 are reduced to produce 1 mol tetraguaiacol.

2.11 β -1,3-glucanase (GLU, EC 3.2.1.39) activity

GLU activity was assayed based on D-glucose liberation from laminarin (Sigma Chemical Company) used as substrate by the action of the enzyme (Boller 1992). The enzyme reaction was assayed in 50 mM Na-acetate buffer, pH 5.2, at 50 ºC. The amount of D-glucose liberated was calculated using a standard curve performed with known D-glucose concentrations varying from 4.1 x 10^4 to 13 x 10^4 nM. The activity was expressed as nanokatal per gram fresh leaf weight (nkat g^{-1} FW). One nkat was defined as 1.0 nmol of D-glucose liberated from laminarin per second under the assay conditions.

2.12 Chitinase (CHI, EC 3.2.1.14) activity

CHI activity of the dialyzed cowpea leaf extracts was conducted as previously described (Boller 1992) by measuring the amount of N-acetyl-D-glucosamine (NAG) (Sigma) produced (Reissig et al. 1995) by the combined hydrolytic action of chitinases and *β*-glucuronidase (Sigma) on non-radioactive colloidal chitin (Molano et al. 1977) used as substrate in the presence of 50 mM sodium acetate buffer, pH 5.2. The amount of NAG produced was calculated using a standard curve built with crescent NAG concentrations from 4.5 to 31.6 nM dissolved in the acetate buffer. CHI activity was expressed as nanokatal per gram fresh leaf weight (nkat g^{-1} FW). One nkat was defined as 1.0 nmol of NAG produced per second at 37 ºC.

2.13 Phenylalanine ammonia-lyase (PAL EC 4.3.1.5) activity

PAL activity was conducted by measuring the amount of trans-cinnamic acid (TCA) generated from L-phenylalanine by the action of the enzyme (Mori et al. 2001, El-Shora 2002). The leaf extract (0.1 mL) was incubated with 0.2 mL 40 mM L-phenylalanine, 0.02 mL 50 mM *β*-mercaptoethanol, and 0.480 mL 100 mM Tris–HCl buffer, pH 8.8, at 30 °C, for 60 min. Reaction was stopped by addition of 0.1 mL 6 x 10^3 mM HCl. After centrifugation (10 000 g, 10 min, 25 °C) the absorbance of the collected supernatant was measured spectrophotometrically at 290 nm. The TCA amount produced was calculated using a standard curve built with known TCA concentrations (66.7 to 10^3 µM) dissolved in 100 mM Tris–HCl buffer, pH 8.8. PAL activity was expressed as the amount of trans-cinnamic acid produced per gram fresh leaf weigh per minute (µmol TCA g^{-1} FW min^{-1}).

2.14 Hydrogen peroxide (H_2O_2) content

Quantitative analysis of H_2O_2 was determined as previously described (Gay et al., 1999). Fresh leaves were detached from the plantlets at 7 DPI, immersed in and powdered with liquid N_2. This fine powder obtained was homogenized in 50 mM borax-borate buffer, pH 8.4, (1:4, m/v), in a mortar and pestle, under an ice bath, for 3 min, centrifuged (12 000 *g*, 4 °C, 20 min), and the supernatant obtained employed in the assay. The reaction mixture consisted of 0.2 mL of the supernatant + 1 mL of xylenol orange prepared by mixing 0.1 mL solution A (250 mM $FeSO_4$ + 250 mM $(NH_4)_2SO_4$ + 2.5 × 10^2 mM H_2SO_4 + 10 mL ultra-pure water) and 10 mL solution B (1.25 mM xylenol orange + 100 mM sorbitol + 100 mL ultra-pure water)]. The reaction mixture was incubated for 30 min at 25 °C, and the absorbances taken at 560 nm. A standard curve (0 – 10.0 nmol H_2O_2/1.2 mL) was performed to determine the leaf H_2O_2 content, expressed as nmol H_2O_2 per gram fresh leaf weight (nmol H_2O_2 g^{-1} FW).

2.15 Phenolic compound content

Extraction and quantification of phenolic compounds were done as previously described (Ainsworth and Gillespie 2007) with minor modifications. Cowpea leaves (1.0 g) were powdered in a mortar and pestle under liquid nitrogen, homogenized with 1.0 mL 95% (v/v) ice-cold methanol and centrifuged at 13 000 *g*, 5 min, at 4 °C. The methanolic extract (0.2 mL) was diluted with 1.0 mL ultra-pure water and 0.4 mL of 1 N Folin-Ciocalteu phenol reagent (Sigma) added. This final mixture was incubated for 5 min at 25 °C, and 0.4 mL 700 mM Na_2CO_2 was added. After 30 min at 37 °C incubation, absorbances were taken at 765 nm. The phenolic content (PC) was expressed in milligram per gram fresh leaf weight (mg PC g^{-1} FW), using gallic acid as reference.

2.16 Lignin detection by microscopy

Lignin detection in the cowpea leaves of CPI, CPU, and MCPI plantlet groups were performed using Phloroglucinol stain (Sigma), according to Mlícková et al. (2004). Fresh leaves were detached at 7 DPI and incubated overnight in 70% (v/v) ethanol containing 1% (w/v) phloroglucinol. Next, the leaves were rinsed in ultrapure water, and bathed in concentrated HCl. After draining excess HCl, leaf pieces were mounted on a microscope glass slide and covered with a glass cover slip in 50% (v/v) glycerol. Light microscopy was performed using an Olympus System Model BX60F5 microscope (Olympus Optical Co. Ltd, Japan). Images were acquired using an Olympus photomicrography system PM-20. Three biological replicates were examined.

2.17 Virus detection by RT-PCR

Total RNA was extracted and purified from cowpea fresh leaves of uninfected and virus infected plantlets at 7 DPI using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany) following the manufacturer's recommendations. RNA quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). To a volume containing 1.0 µg of the purified RNA, 10^{-3} mL oligo(dT_{15}) (Promega)

and RNase-free water were added to complete 1.16 x 10^{-3} mL volume. This mixture was incubated in a thermocycler (PTC 200-MJ Research, USA) for 5 min at 70 °C, and 5 min at 4 °C. Next 4 x 10^{-3} mL of the enzyme Improm II buffer, 10^{-3} mL of 1.0 mM dNTPs, 2.4 x 10^{-3} mL of 25 mM $MgCl_2$, and 10^{-3} mL of the reverse transcriptase Improm II (1000 unit/mL, Promega) were added to 20 x 10^{-3} mL final reaction volume. RT-PCR reaction was programmed as follows: 25 °C for 5 min; 42 °C for 60 min; 70 °C for 15 min; and a final incubation at 4 °C. The reaction products were storage at -20 °C until further analysis.

To confirm the absence and/or the presence of CPSMV in the uninfected and virus infecting cowpea plantlets, the first-strand cDNA product that correspond to a fragment of the viral coat protein gene obtained was amplified by RT-PCR using two degenerated universal oligonucleotide primers for the Comovirus genus (Brioso et al. 1996): 5'-GCATGGTCCACWCAGGT-3'(forward) and 5'-YTCRAAWCCVYTRTTKGGMCCACA-3' (reverse). The RT-PCR reaction thermal cycler programmed was an initial denaturation step at 95 °C for 5 min, 40 cycles consisting of denaturation at 94 °C for 20 sec, annealing at 41 °C for 20 sec, and extension at 72 °C for 45 sec, followed by an extension step at 72 °C for 10 min. Upon completion of the reaction, the products were visualized (ethidium bromide-staining) after 2% (m/v) agarose gel electrophoresis run carried out in a Pharmacia Biotec electrophoresis unit for 40 min, at 25 °C, 100 V, 50 mA.

2.18 Statistical analyses

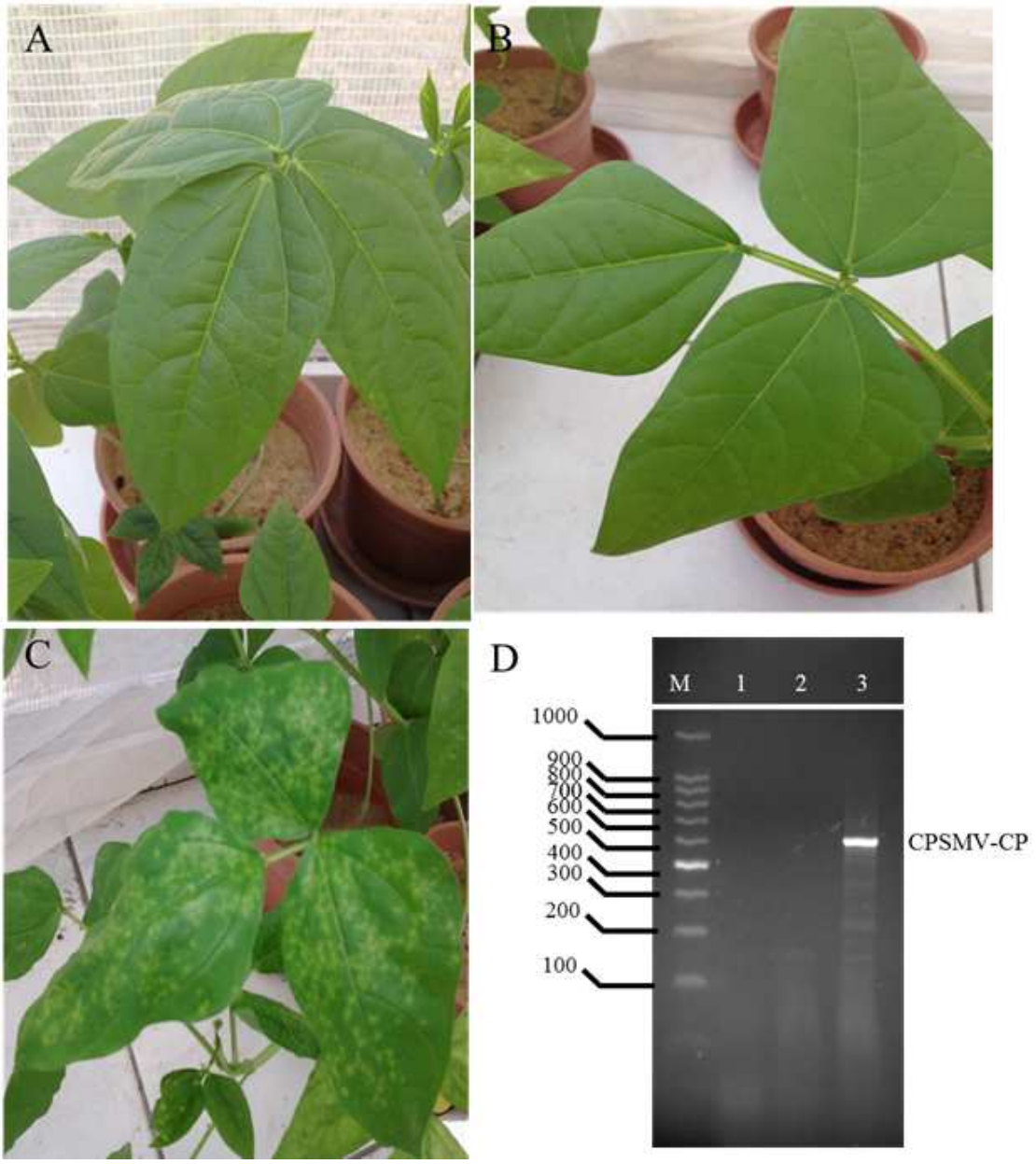
Data from the physiological parameters, enzyme assays and metabolite contents were compared using one-way analysis of variance (ANOVA) followed by the Tukey's test ($\rho \leq 0.05$).

3 Results

3.1 Viral infection symptoms

Severe mosaic and chlorosis were typical symptoms observed in the cowpea leaves of CPI plantlets at 7 DPI (Fig. 1C). However they were absent in the leaves of the mutagenized resistant cowpea plantlets (MCPI) grown from the CE-31 seeds that were previously treated with the mutagenic agent EMS, similarly infected with the CPSMV isolate (Fig. 1B). Obviously, these disease symptoms were also absent in the plantlet group (CPU), originated from the non-mutagenized CE-31 seeds, that were not inoculated with CPSMV (Fig. 1A). Indeed, RT-PCR analysis confirmed the unique presence of the cDNA fragment (Fig. 1D – Lane 3) of the virus coat protein in the compatible interaction (CPI), but neither in CPU or in the MCPI plantlet group, confirming the absence of the virus (Fig. 1D – Lane 1 and 2).

Fig. 1. Leaf morphological appearance of the CPSMV-susceptible cowpea CE-31 genotype (A and C) and its derived EMS-mutagenized (MCPI) resistant (B) cowpea after CPSMV inoculation, and CPSMV indirectly detection by RT-PCR. (A) CPU; (B) MCPI; and (C) CPI plantlets, all examined at 7 DPI with CPSMV. (D) 1.5% (m/v) agarose gel electrophoresis of RT-PCR products. Lane M: Molecular weight marker; Lane 1, 2, and 3: Amplified cDNA fragment of CPSMV-coat protein from CPU, MCPI and CPI leaf samples, respectively.



3.2 Physiological parameters, chlorophyll and carotenoid contents

Challenge of the susceptible CPI plantlet with CPSMV led to significantly ($\rho \leq 0.05$) alterations of photosynthetic parameters in attached leaves at 7 DPI, compared with the mock inoculated plantlets (CPU). The net CO_2 assimilation rate (P_N), effective quantum efficiency of PSII ($\Delta F/Fm'$), and non-photochemical quenching (NPQ) (Fig. 2A, C, D) decreased 61.2%, 49.3%, and 50.8%, respectively, in relation to CPU group. However, the stomatal conductance (gs) was similar between these two plantlet groups (Fig. 2B). On the other hand, MCPI plantlet group did not show any significant alteration of these above parameters (Fig. 2A, C and D), except in gs that was higher (76%) as compared with the CPU plantlets (Fig. 2B).

Data of P_N, gs, $\Delta F/Fm'$, and NPQ for MPCI compared with those of PCI plantlets (Fig. 2) were significant ($\rho \leq 0.05$) higher (77.9%, 92.8%, 51.1%, and 41.1%, increases, respectively), suggesting that the virus infection affect the photosynthesis-related process in susceptible (CPI) plantlets. Other physiological parameters, such as internal pressure of CO_2 (Ci), maximum efficiency of PSII (Fv/Fm), and photochemical quenching (qP) were not affected (Fig. 3).

In agreement with the observed changes in the photosynthesis-related processes induced by CPSMV infection, a significant ($\rho \leq 0.05$) decrease in the total chlorophyll (32.3%) and chlorophyll *a* (39.7%) contents in CPI, compared to CPU (Fig. 4A and B), were noted. Contrarily, the total chlorophyll and chlorophyll *a* contents of MCPI increased significantly ($\rho \leq 0.05$) (13.3% and 31.8%, respectively) compared with CPU, and 50.0% and 84.2%, respectively, compared with CPI. However, the chlorophyll *b* contents of CPI and MCPI decreased significantly (46.3% and 47.5%, respectively) in comparison with CPU (Fig. 4C). Regarding to carotenoid, its content in MCPI was 51.1% and 79.2% higher, respectively, compared with those of CPU and CPI (Fig. 4D).

Fig. 2. Effect of CPSMV inoculation on net CO_2 assimilation (A), stomatal conductance (B), effective quantum efficiencies of PSII (C), and non-photochemical quenching (D) in cowpea leaves at 7 DPI with CPSMV. Control-uninoculated (CPU) leaves were used as controls. Each point represents the mean of three biological replicates (± SD). Bars followed by the same small letters are not significantly different at $p \leq 0.05$. ▭ CPU; ▥ CPI; ▩ MCPI.

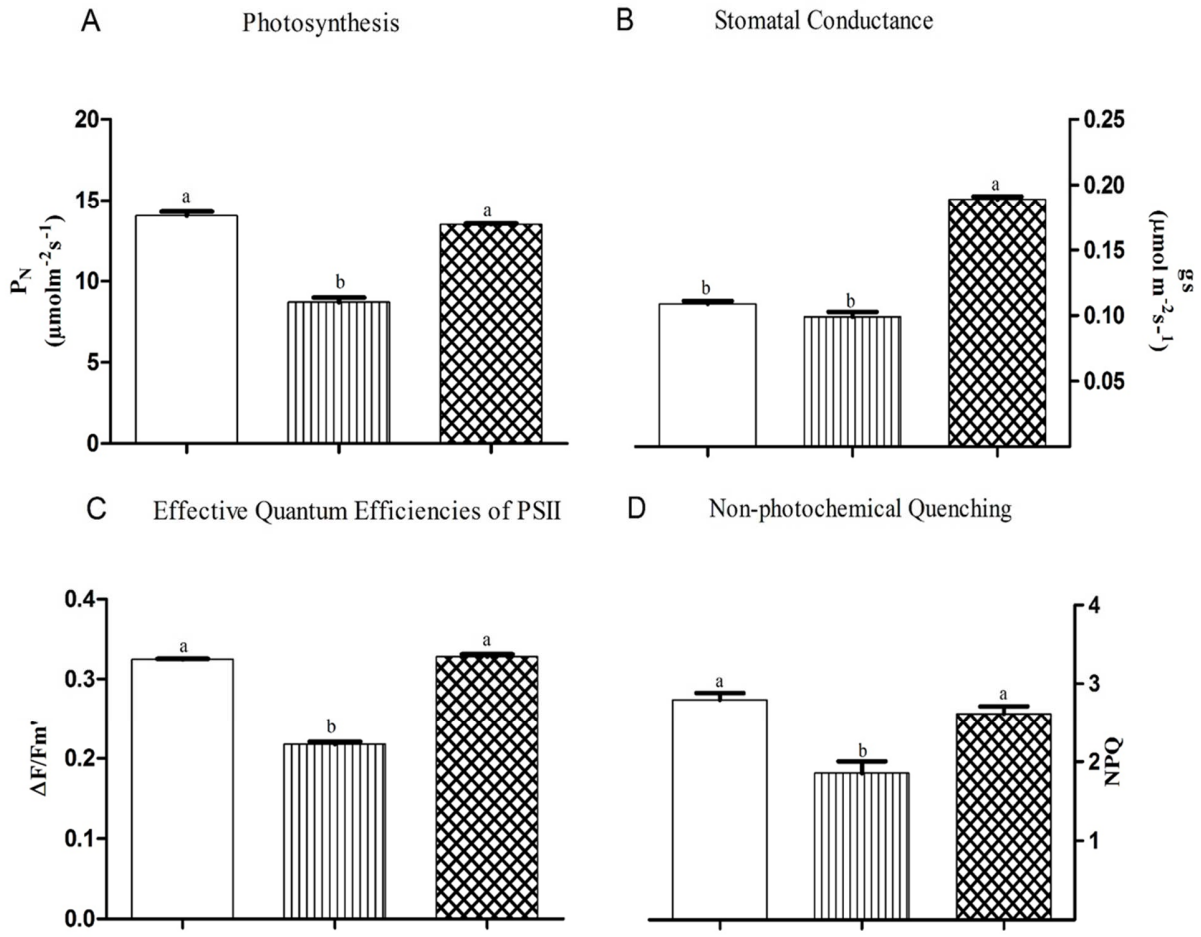


Fig. 3. Effect of CPSMV inoculation on internal CO_2 pressure, maximum quantum efficiencies of PSII and photochemical quenching of cowpea leaves. CPU; CPI; MCPI.

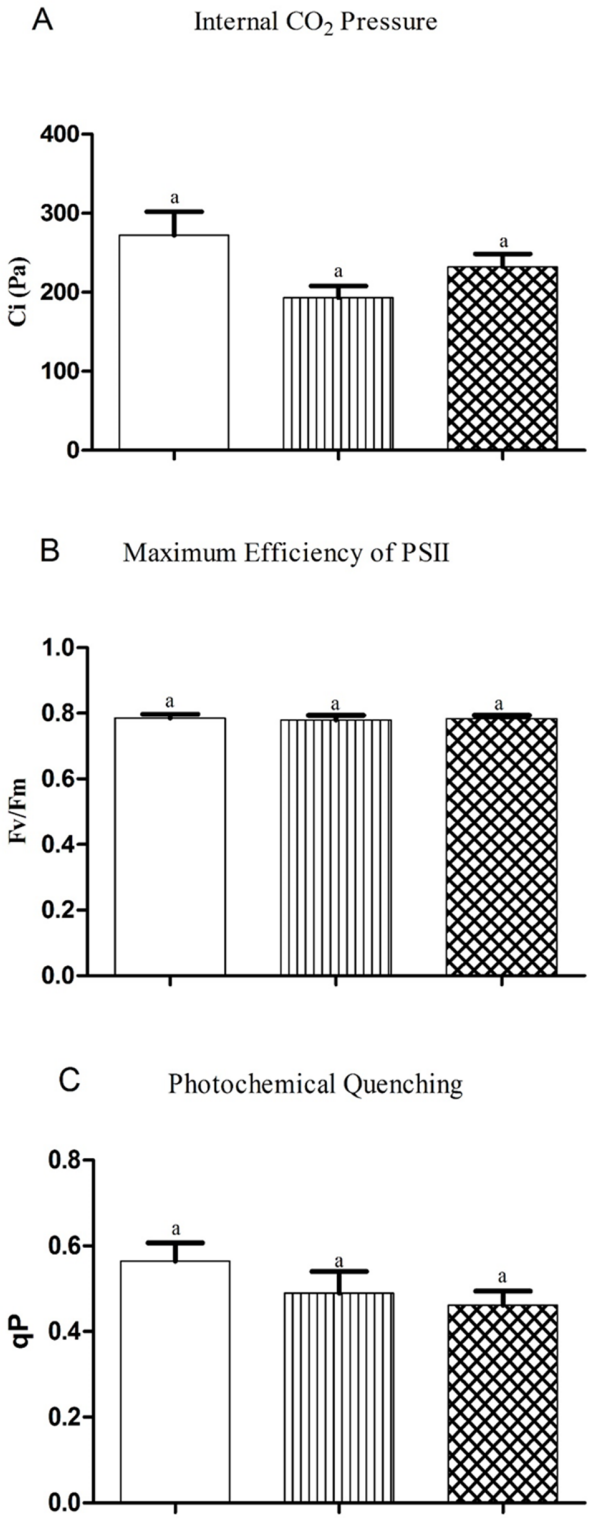
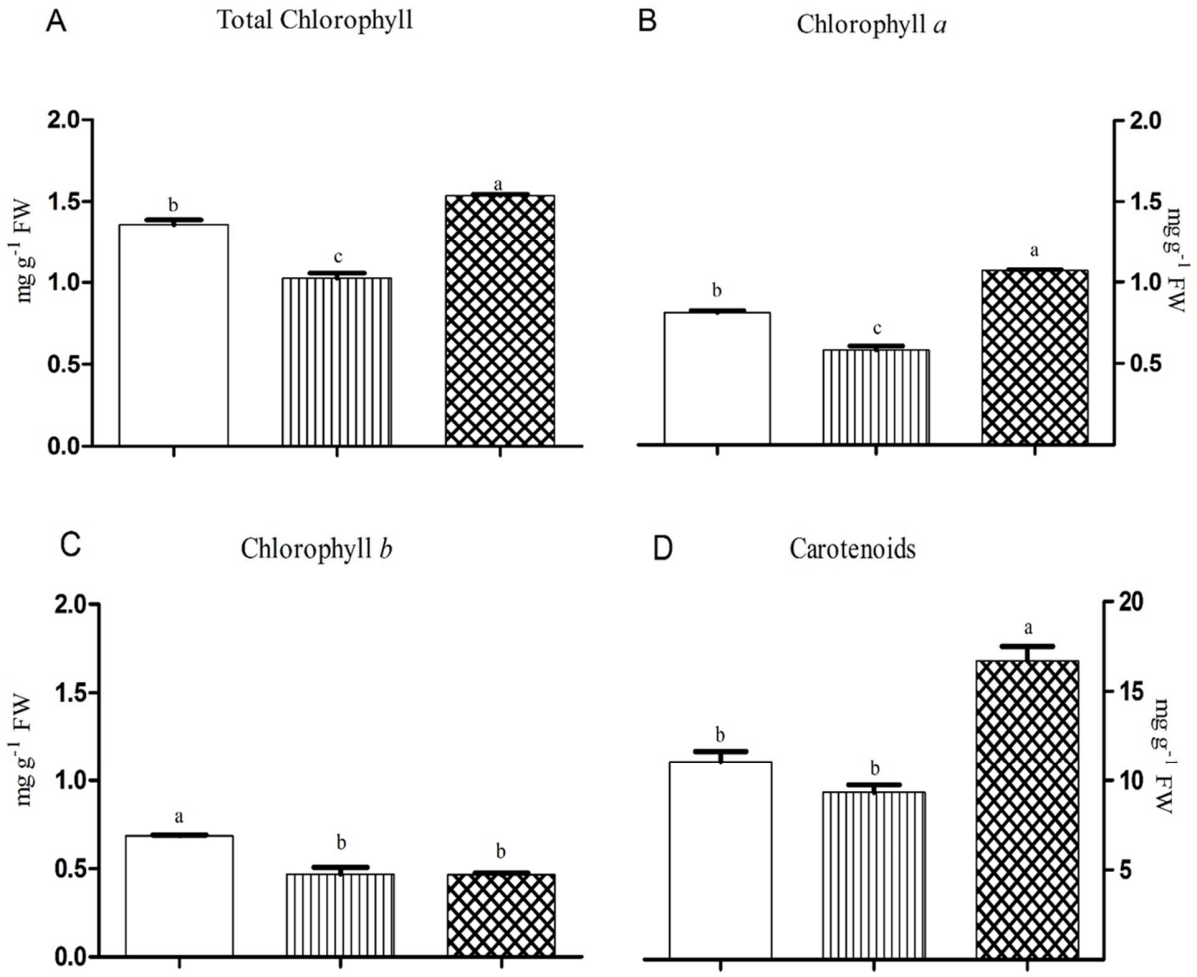


Fig. 4. Effect of CPSMV inoculation on total chlorophyll (A), chlorophyll *a* (B), chlorophyll *b* (C), and carotenoid (D) contents in cowpea leaves at 7 DPI with CPSMV. Control-uninoculated (CPU) leaves were used as controls. Each point represents the mean of three biological replicates (± SD). Bars followed by the same small letters are not significantly different at $p \leq 0.05$. ▭ CPU; ▥ CPI; ▩ MCPI.



3.3 Soluble protein contents, PR-proteins, and PAL activity of CPU, CPI, and MCPI

The soluble leaf protein content of MCPI (3.77 ± 0.07 mgP g^{-1} FW) was significant ($\rho \leq 0.05$) lower (29.1%) than in CPI (4.87 ± 0.12 mgP g^{-1} FW), at 7 DPI, but higher (22.5%) than that of CPU (2.92 ± 0.12 mgP g^{-1} FW) (Fig. 5A).

The PAL activity (Fig. 5B) of MCPI (7.46 ± 0.33 µmol TCA g^{-1} FW min^{-1}) was 894.7% and 234.5% higher at 7 DPI, compared, respectively, to CPU (0.75 ± 0.05 µmol TCA g^{-1} FW min^{-1}) and CPI (2.23 ± 0.19 µmol TCA g^{-1} FW min^{-1}). Similarly to MCPI, PAL in CPI was also induced upon CPSMV infection, but at less extension, as 197.3% higher in comparison to CPU. Therefore these data indicated that PAL is responsive to CPSMV infection, although much expressively higher in the CPSMV-resistant MCPI plantlets.

The chitinase activity of MCPI at 7 DPI was 0.078 ± 0.008 nkat g^{-1} FW. This value was 62.5% and 44.4% higher than the data found for CPI (0.048 ± 0.004 nkat g^{-1} FW) and CPU (0.054 ± 0.003 nkat g^{-1} FW), respectively (Fig. 5C). However, no significant difference of CHI activity was detected between CPI and CPU. Related to the GLU activity of the resistant MCPI plantlets (0.80 ± 0.037 nkat g^{-1} FW) no significant difference was observed in relation to that of CPU (0.76 ± 0.008 nkat g^{-1} FW) (Fig. 5D). However, the GLU activity of CPI (1.07 ± 0.047 nkat g^{-1} FW) was 33.7% and 40.8% higher, respectively, as compared to both MCPI and CPU plantlets.

3.4 Cell homeostasis redox proteins

The SOD activity (5.62 ± 0.80 AU g^{-1} FW) in the leaves of the mutagenized resistant plantlets (MCPI) was 111.3% higher than that of CPI (2.66 ± 0.11 AU g^{-1} FW) at 7 DPI (Fig. 6A), which, in turn, presented a SOD activity significant ($\rho \leq 0.05$) higher (50.3%) than that of CPU (1.77 ± 0.23 AU g^{-1} FW) at the same time point.

In relation to the CAT activity of MCPI (23.44 ± 1.443 µmol H_2O_2 g^{-1} FW min^{-1}) it was 74.1% and 64.3% lower than those of CPI (84.26 ± 12.320 µmol H_2O_2 g^{-1} FW min^{-1}) and CPU (61.54 ± 7.313 µmol H_2O_2 g^{-1} FW min^{-1}), respectively, at 7 DPI **(**Fig. 6B).

The GPOX activity of the mutagenized resistant MCPI (248.035 ± 26.54 µmol H_2O_2 g^{-1} FW min^{-1}) was 173.2% and 73.1% higher than the respective values for CPI (109.052 ±

8.17 µmol H_2O_2 g^{-1} FW min^{-1}) and CPU (172.07 ± 0.50 µmol H_2O_2 g^{-1} FW min^{-1}) plantlets at 7 DPI (Fig. 6C). Moreover, the GPOX activity of CPI was 57.8% lower than that of CPU.

Challenged of the cowpea plantlets with the virus isolate CPSMV led to a significant ($\rho \leq 0.05$) decrease of the APX activity (Fig. 6D), irrespective whether in MCPI (0.818 ± 0.132 µmol H_2O_2 g^{-1} FW min^{-1}) or CPI (1.73 ± 0.203 µmol H_2O_2 g^{-1} FW min^{-1}) as compared with CPU (3.275 ± 0.470 µmol H_2O_2 g^{-1} FW min^{-1}) at 7 DPI. However, decrease of APX in MPCI (77%) was much more accentuated than in CPI (59.1%) indicating that CPSMV inoculation caused a much drastic negative regulation of APX activity in the MCPI plantlets.

3.5 H_2O_2, phenolic compound, and lignin contents

In consonance with increase in SOD activity, quantitative analysis showed that the H_2O_2 content of the mutagenized resistant MCPI (1.50 ± 0.02 0.178 ± 0.01 nmol g^{-1} FW) had an increase of 275% and 742%, respectively, in comparison to that of CPI (0.40 ± 0.01 nmol g^{-1} FW) and CPU (0.178 ± 0.01 nmol g^{-1} FW) (Fig. 6A).

In agreement with the highest PAL activity (Fig. 5B**)** in MCPI compared to both CPI and CPU, the phenolic content in the MCPI leaves (15.28 ± 0.02 mg PC g^{-1} FW) was 54.5% and 63.9% higher, respectively, compared to that of CPI (9.89 ± 0.01 mg PC g^{-1} FW) and CPU (9.32 ± 0.01 mg PC g^{-1} FW) (Fig. 6B). In addition to increased PAL (Fig. 5B) and GPOX (Fig. 6C) activities, leaf lignifications in MCPI (Fig. 8C) was higher than in both CPU and CPI, at 7 DPI (Fig. 8).

Fig. 5. Effects of CPSMV inoculation on total soluble protein (A) and PAL (B), CHI (C), and GLU (D) activities in cowpea leaves at 7 DPI with CPSMV. Control-uninoculated (CPU) leaves were used as controls. Each point represents the mean of three biological replicates (± SD). Bars followed by the same small letters are not significantly different at $p \leq 0.05$. ▭ CPU; ▥ CPI; ▩ MCPI.

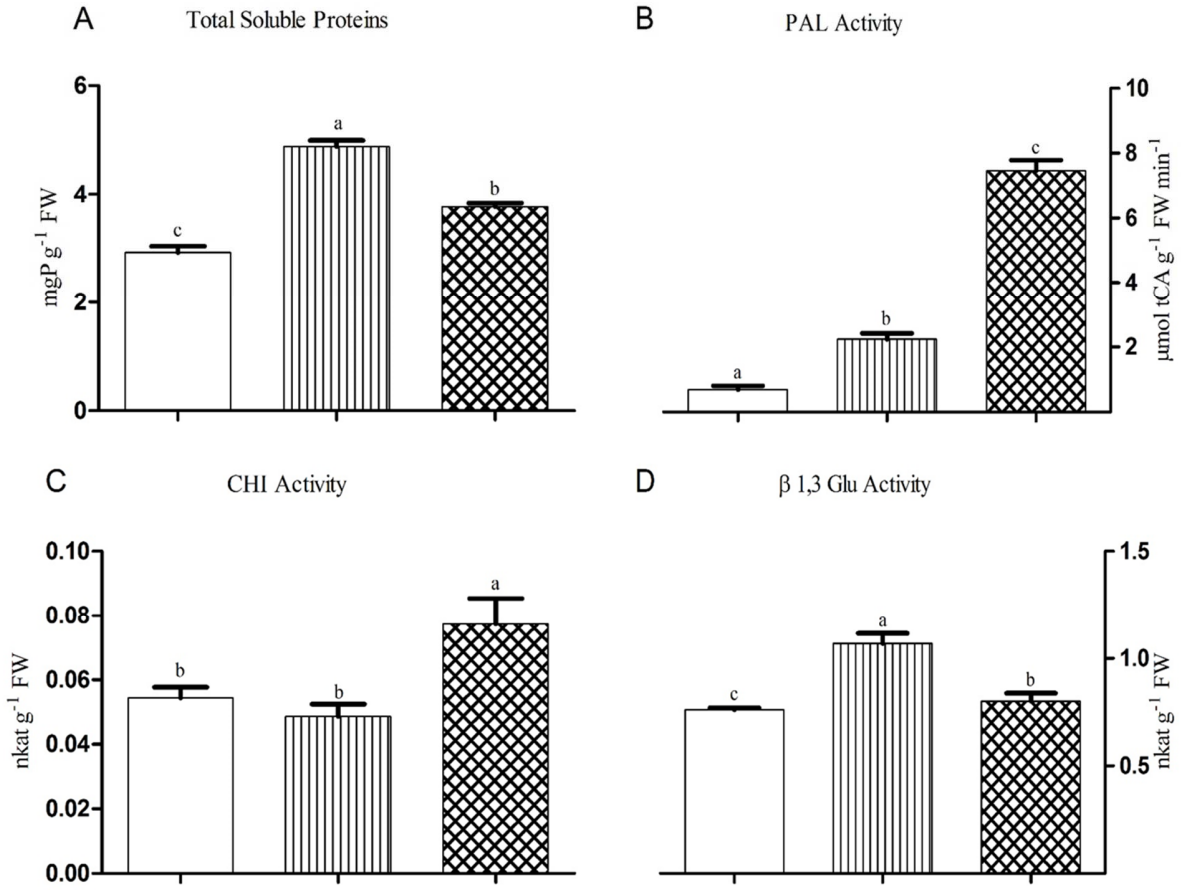


Fig. 6. Effects of CPSMV inoculation on SOD (A), CAT (B), GPOX (C), and APX (D) activities in cowpea leaves at 7 DPI with CPSMV. Control-uninoculated (CPU) leaves were used as controls. Each point represents the mean of three biological replicates (± SD). Bars followed by the same small letters are not significantly different at $p \leq 0.05$. CPU; CPI; MCPI.

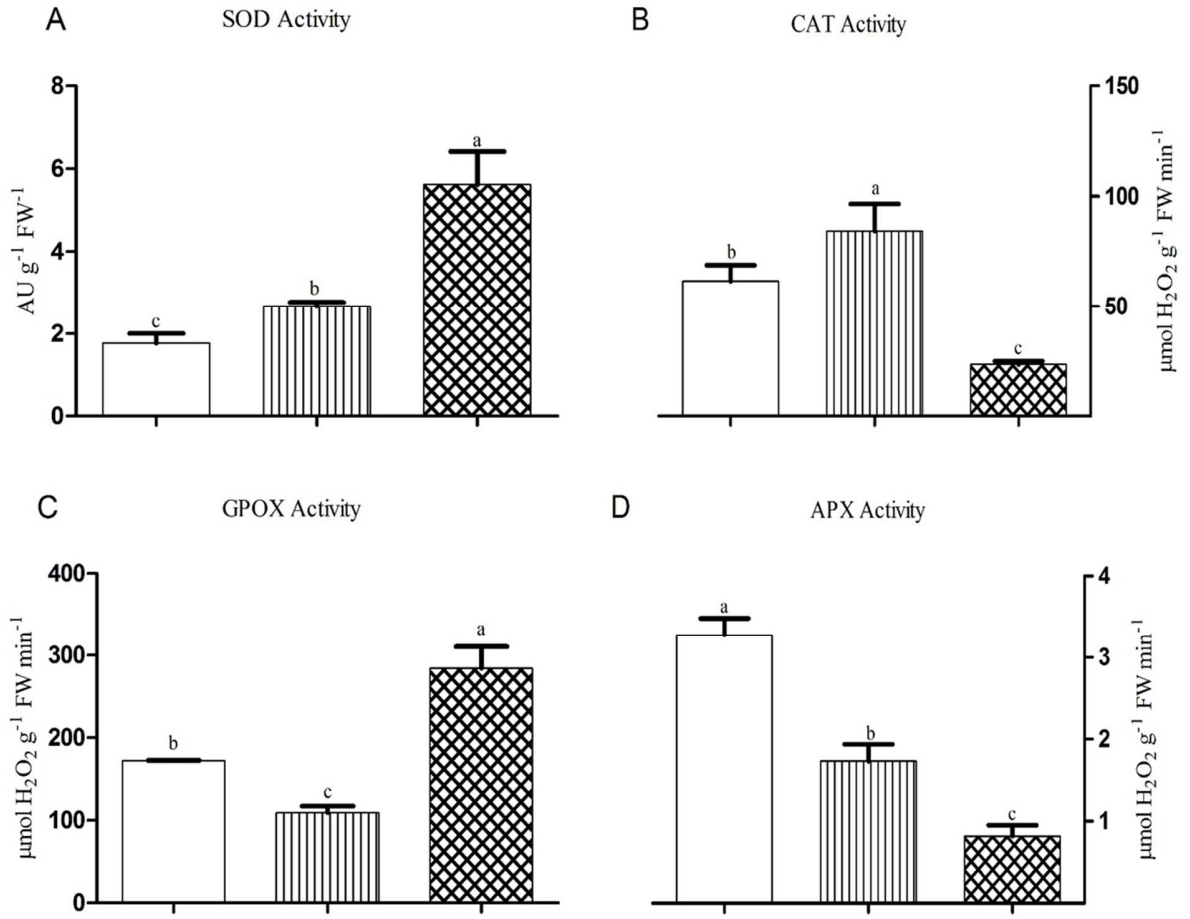


Fig. 7. Changes in the contents of H_2O_2 (A) and phenolic compounds (B) in cowpea leaves at 7 DPI with CPSMV. Control-uninoculated (CPU) leaves were used as controls. Each point represents the mean of three biological replicates (± SD). Bars followed by the same small letters are not significantly different at $p \leq 0.05$. ▭ CPU; ▥ CPI; ▩ MCPI.

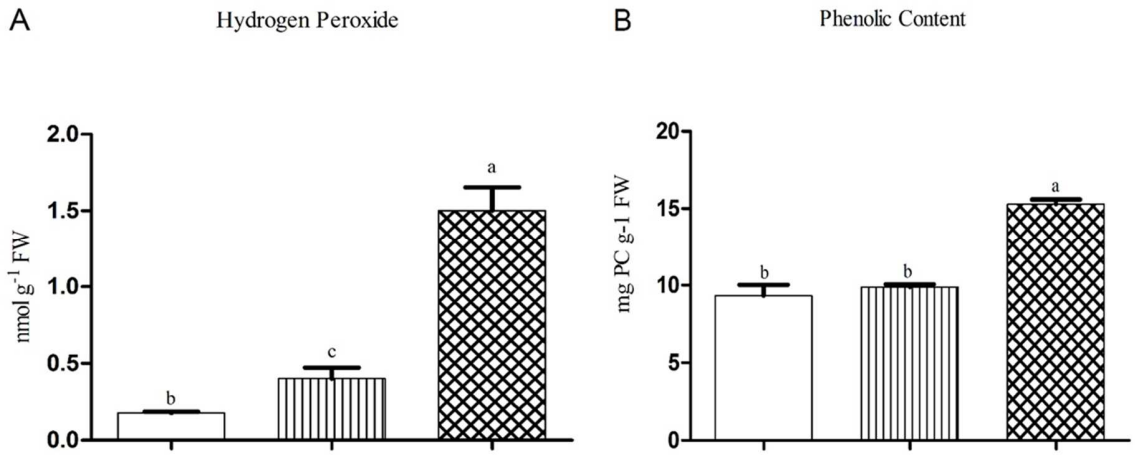
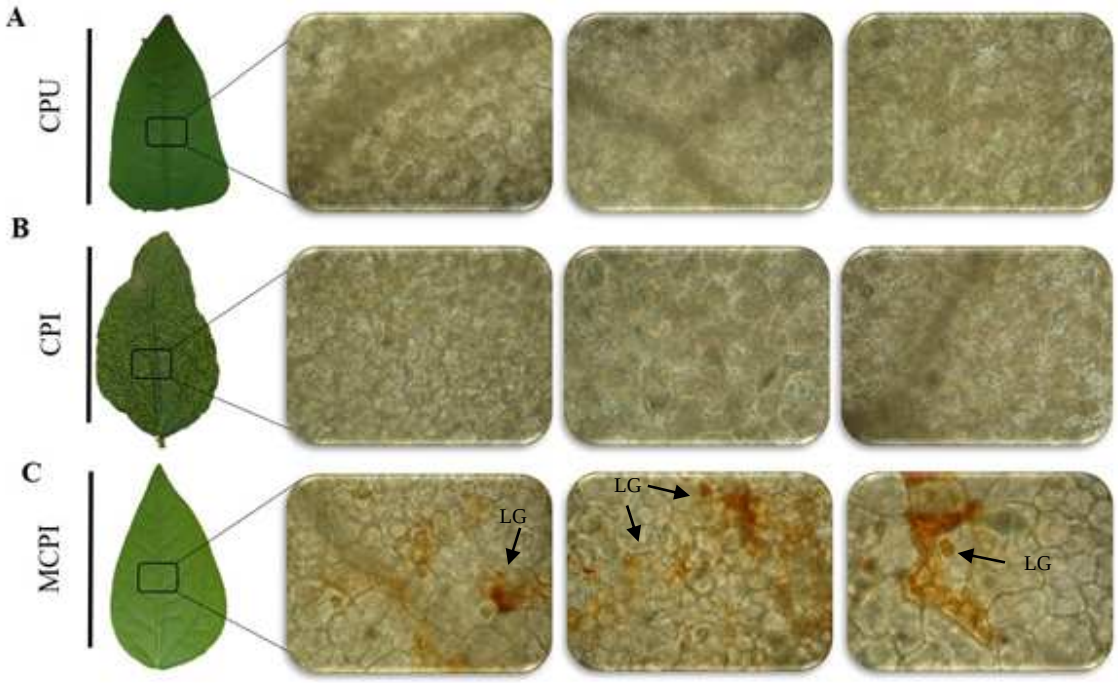


Fig. 8. Lignin (LG) accumulation (arrow) in the cowpea leaves at 7 DPI with CPSMV. (A) Control-uninoculated cowpea (CPU) plantlets; (B) Inoculated cowpea plantlets (CPI); (C) Mutagenized resistant cowpea plantlets (MCPI). Leaves were stained with 70% (v/v) ethanol containing 1% (m/v) phloroglucinol. Experiments were performed thrice with three biological replicates.



4. Discussion

4.1 Virus challenge and CPSMV inoculation

The main symptoms that appear when a susceptible plant is infected by viruses are mosaic formation, leaf deformation, foliar necrosis, and, in extreme cases, death. One kind of host resistance against viruses is associated with the plant ability to recognize the pathogen and evoke proteins and secondary compounds to avoid systemic virus spread, preventing appearance of symptoms and disease establishment (Laliberté and Zheng 2014, Soosaar et al. 2005).

In this current work, the CPI plantlets suffered severe disease symptoms and accumulation of the coat protein gene of CPSMV (CPSMV-CP) (Fig. 1C, D Lane - 3), because the virus successfully replicated in the plant tissues. On the other hand, in MCPI, the absence of CPSMV-CP agrees with the absence of the typical disease symptoms, providing evidence that CPSMV failed to replicate in MCPI (Fig. 1B, D – Lane 2). This phenomenon of EMS-induced resistance, previously verified in *A. thaliana* seeds that became resistant to TuMV after EMS-treatment was called *loss-of-susceptibility* as the mutagenized plantlets were devoid of TuMV symptoms infection (Lellis et al. 2002).

4.2 Changes in photosynthetic parameters, chlorophyll, and carotenoid content induced by CPSMV

Photosynthesis is the most important metabolic process in plants, in which light energy is converted to chemical energy (ATP), reduced power (NADPH), metabolic intermediates and organic compounds (Scharte et al. 2005). Viral infection in many susceptible plants induces reduction in photosynthesis-related process (CO_2 assimilation and chlorophyll fluorescence parameters) (Gonçalves et al. 2005, Rys et al. 2014). Photosynthesis also plays an important role in plant defense responses to pathogen attack because this process induces ROS production in the chloroplasts, which can diffuse to cytosol and hinder viral spreading. In addition, photosynthesis can also act as an energy source to cells to cope with infection. Thus, reduction in photosynthesis

induced by biotrophic pathogens, like viruses, could be one the primary strategies to establish a compatible interaction with plant hosts (Bolton 2008). Hodgson et al. (1989) demonstrated that TMV (*Tobacco Mosaic Virus* genus *Tobamovirus*) infection in spinach (*Spinucia olerucea*) led to TMV-CP accumulation at the PSII complex in thylakoids and consequent specific inhibition of electron transport within and from PSII, which prevents the conversion of absorbed light energy into electrochemical energy thus inducing photosynthesis inhibition.

Here, we demonstrated that CPSMV infection in cowpea susceptible plantlets (CPI) causes a significant reduction in the net CO_2 assimilation, effective quantum efficiencies of PSII, and NPQ in cowpea leaves (Fig. 2), as well as decrease in the contents of chlorophyll *a*, chlorophyll *b*, and total chlorophyll at 7 DPI (Fig. 4). It is interesting to note that in spite of CPSMV infection in CPI had affected PSII activity (indicated by decreases in ∆Fv/Fm', Fig. 2C), it apparently did not affect the PSII integrity, since no change was observed in Fv/Fm (Fig. 3). The potential quantum efficiency of photosystem II (Fv/Fm) of sugarcane (*Saccharum* spp.) was reduced upon infection with ScYLV (*Sugarcane Yellow Leaf Virus* genus *Polerovirus*), suggesting that ScYLV leads to PSII destabilization owing to the prolonged over-reduction of the photosynthetic electron transport (Gonçalves et al. 2005). In addition, ScYLV infection also led to CO_2 assimilation reduction in sugarcane because the virus might have interfered on one or more steps of CO_2 assimilation process (Gonçalves et al. 2005). Contrarily, in this current work, MCPI did not suffer reduction in the photosynthesis process, which may have also contributed to the resistance trait of these chemically induced mutagenized resistant plantlets.

Interestingly, NPQ levels strongly decreased in CPI, whereas in MCPI no reduction was detected at 7 DPI (Fig. 2D) in relation to CPU. Apparently, decreased NPQ favors CPSMV infection. NPQ is a complex mechanism, which reflects different processes occurring in the plant chloroplasts. The most rapid component of NPQ, called qE, is dependent on delta-pH formation and photoprotective mechanisms to dissipate excess light energy (NPQ) captured by the PSII complex as heat. Indeed, increase in NPQ results in the augment of heat production, which have been shown to accompany ROS accumulation in TMV-inoculated resistant tobacco leaves, probably by increased oxygen uptake (Chaerle et al. 1999). For instance, increased NPQ was associated with the leaf

areas invaded by PMMoV (*Pepper Mild Mottle Virus* genus *Tobamovirus*) (Pérez-Bueno et al. 2006).

CPSMV infection provoked a significant decrease in total chlorophyll, and chlorophyll *a* in CPI compared to MCPI (Fig. 4A, and B). These results suggest that MCPI blocked somehow the breakdown of chlorophyll and/or induced its biosynthetic reactions. Maintenance of chlorophyll levels is important for chloroplasts to produce energy and ROS to allow the plant to counterattack CPSMV infection (Liu et al. 2014). Several works (Kundu et al. 2013, Rys et al. 2014) have demonstrated that in compatible interactions between plants and viruses the chlorophyll content is strongly reduced. Reduction in chlorophyll content associated with virus infection might be due to chlorophyll degradation (Liu et al. 2014) or inhibition of chlorophyll synthesis (Shimura et al. 2011). During the compatible interaction between Cassava (*Manihot esculenta* Crantz) and ACMV (*African Cassava Mosaic Virus* genus *Begomovirus*), the mosaic phenotype coincide with the upregulation of chlorophyll degradation-related genes and 30% reduction in the chlorophyll content when compared with healthy plants (Liu et al. 2014). In contrast, Shimura et al. (2011) revealed that susceptible tobacco plants infected with CMV-YsatRNA presented a specific interfering RNA event caused by Y-sat sequence, which interfere with translation of plant mRNA of tobacco magnesium protoporphyrin chelatase subunit I (*ChlI*, the key gene involved in chlorophyll synthesis), thus inducing the specific chlorosis and mosaic symptoms. In both studies, the authors suggest that impairment in chlorophyll metabolism (degradation or synthesis inhibition) induced by virus infection directly contributes to the development of the viral disease symptoms.

MCPI showed a highly increase in the carotenoid content in comparison with CPU and CPI (Fig. 4D). Kundu et al. (2013) reported similar results in plant-virus incompatible interaction between mungbean (*Vigna mungo*) and MYMIV (*Mungbean Yellow Mosaic India Virus* genus *Begomovirus*) at 7 DPI. Carotenoids have no direct action on plant defense against viruses. However, they are antioxidants that protect chloroplasts from damage caused by ROS and act as membrane stabilizers of the lipid phase of the thylakoid membranes in chloroplasts (Havaux 1998). Interactions between carotenoids and membrane lipids decrease membrane fluidity, increased membrane thermostability, and lowered susceptibility to lipid peroxidation (Havaux 1998). Therefore, the higher

carotenoid content in MCPI possibly acts as antioxidant, protecting the photosynthetic apparatus against damage caused by ROS. The maintenance of the photosynthetic-related process in MCPI suggests that the increased photosynthetic process (increased photochemistry activity, CO_2 assimilation rates, and photosynthetic pigment contents) in these mutagenized plantlets may be related to resistance, because photosynthesis might provide energy (ATP) and reducing potential (NADPH) to support the primary metabolism allowing resistant plants to counterattack virus infection.

4.3 Total soluble proteins and Pathogenesis-related proteins

Involvement of proteins in plant defense has been well documented in several plant-pathogen interactions. Proteins are responsible for pathogen recognition (R proteins), cellular signaling, defense signal amplification, and effective defense response to pathogen attacks (Jones and Dangl 2006**)**. In our study, the total soluble protein content of CPI was higher than that of MCPI which, in turn, was higher than that of CPU (Fig. 5A). In other words, cowpea plants elevated the overall protein content upon virus-infection, irrespective whether susceptible or resistant to CPSMV.

Significantly increased CHI activity in MCPI was observed in comparison with CPU and CPI, at 7 DPI. On the other hand, GLU activity was higher in CPI that in CPU and MCPI (Fig. 5C, D). Other studies indicated that GLU and CHI activities were strongly induced in plant defense response to fungi and bacteria than in compatible interactions between plants and viruses (Kim and Hwang 1994). However, the role of these enzymes in plant-virus pathosystems is unclear. Elvira et al. (2008) reported that in the incompatible interaction between pepper (*Capsicum chinense*) and the Spanish strain of PMMoV four chitinase isoforms increased in abundance, whereas in the compatible interaction between pepper and the Italian strain the chitinase activity did not change. These contradictory results emphasize the importance to further study plant chitinases in the interaction between viruses and plants to definitively establish their target.

Concerning to GLU, it is known that the increased GLU activity in plants infected with viruses is favorable to cell-to-cell movement of viruses via plasmodesmata (Iglesias and Meins, 2000). GLU deficient tobacco plants by antisense suppression exhibited delayed

in the spread of both TMV and a recombinant PVX (*Potato Virus X* genus *Potexvirus*) (Iglesias and Meins, 2000). In this work, we reported that CPU and MCPI showed lower GLU activity than CPI, indicating that higher GLU activity also contributes to the CPI susceptibility to CPSMV.

4.4 PAL activity and phenolic compounds

PAL is a key enzyme of the general phenylpropanoid pathway involved directly of indirectly in the biosynthesis of phenolic compounds, phytoalexins, lignins ant others secondary compounds. For example, PAL inhibition or low activity affects directly the phenolic compound biosynthetic pathway (Chaman et al. 2003). Increased PAL activity has been reported in resistant plants in response to virus infection (Siddique et al. 2014). In this current study, significant increased on PAL activity was noticed in MCPI in comparison with CPU and CPI (Fig. 5B). Concurrently MCPI displayed increased high phenolic content (Fig. 6B) and lignin accumulation (Fig. 8C). Resistant cotton (*Gossypium herbaceum*) plants to CLCuBuV (*Cotton Leaf Curl Burewala Virus* genus *Begomovirus*) infection presented 13.48% increase in PAL activity and increased (21.11%) phenolic content compared with healthy susceptible cotton plants (Siddique et al. 2014). Phenolic compounds generate lignin precursors (coumaryl, synapil, coniferyl) and thus plant cell wall lignifications to form a physical barrier that blocks the pathogen spreading through the plant tissues (Ngadze et al. 2012). Thus increased PAL activity combined with increased GPOX activity (which takes part in the lignin biosynthesis) in MCPI might have led to lignin accumulation (Fig. 8C), as PAL and GPOX work together in lignin biosynthesis pathway (Passardi et al. 2004, Vanholme et al. 2010). Probably, the correlation between PAL and GPOX activity and phenolic compounds (total phenolic compounds and lignin) is vital to MCPI plantlets restrict CPSMV infection and systemic disease establishment.

4.5 Redox homeostasis and H_2O_2 content

During viral infection, ROS accumulation over that of physiological levels can act as a signal molecule leading to the programmed cell death (Jones and Dangl 2006) to avoid viral spreading to the plant vascular tissue and hence systemic infection. ROS also induce PR-protein gene expression (Torres 2010). However, ROS overproduction has to be transient because unbalance between generation and scavenge results in oxidative damage to proteins, nucleic acids, chloroplasts, and other organelles, and promotes lipid peroxidation. In plants the antioxidant enzymes SOD, CAT, APX, and GPOX act toward controlling the redox homeostasis in cellular compartments (Clarke et al. 2002, Gill and Tuteja 2010).

MCPI showed high SOD and low CAT activity, whereas CPI have low SOD and high CAT activity at 7 DPI (Figs. 6A and 6B). This led to a transient increased in H_2O_2 levels in MCPI compared to CPI (Fig. 6A). Kundu et al. (2013) also reported increased SOD activity and declined CAT activity in mungbean (*V. mungo*) resistant to MYMIV infection and also higher H_2O_2 content at 7 DPI. High H_2O_2 content is involved in direct pathogen destruction, PCD, and expression of defense genes in plant cells (Barna et al. 2012).

Concerning to increase in CAT activity in CPI plantlets, several works reported that CAT enhances virus infection during compatible interactions (Krzymowska et al. 1997). Krzymowska et al. (1997) showed that increase in CAT activity reduced H_2O_2 level resulting in severe local disease symptoms, favoring TMV infection of tobacco plants. Similarly, increased CAT activity in CPI, at 7 DPI, probably, favored CPSMV infection. Host gene manipulation by viruses as a strategy towards effective infection has been observed in plant-virus interactions (Laliberté and Zheng 2014).

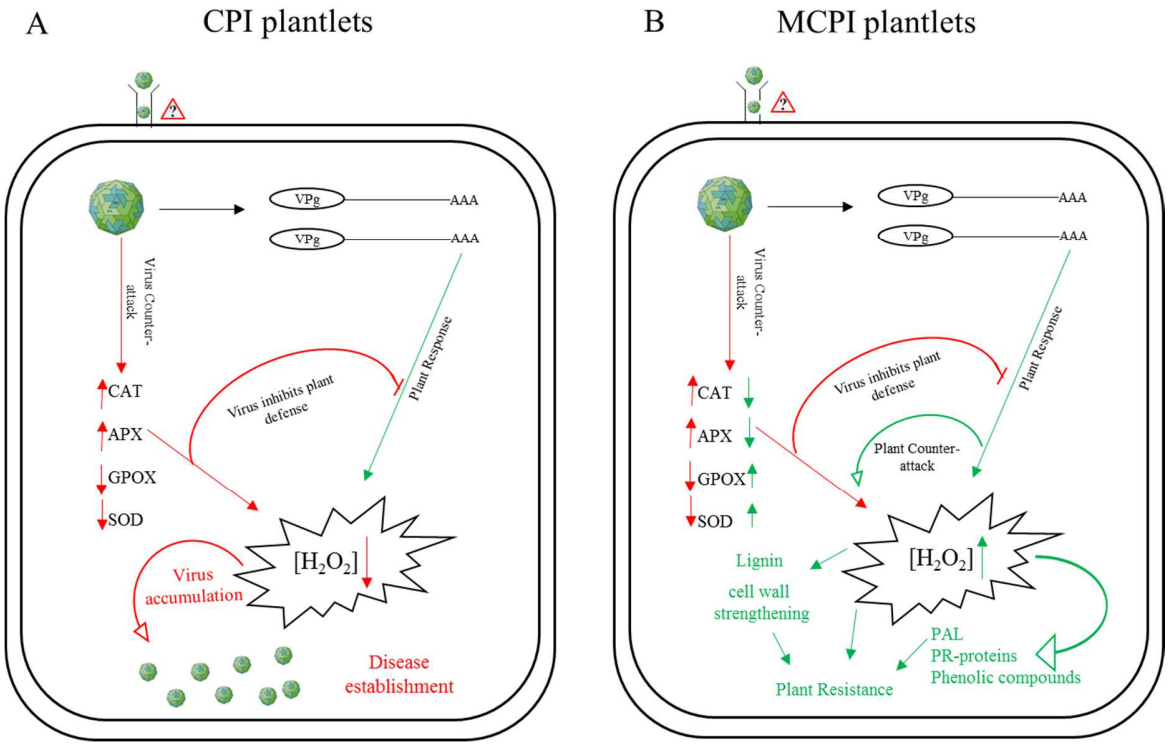
Increased GPOX activity in MCPI compared with CPU and CPI plantlets was demonstrated (Fig. 6C) in our work. Gonçalves et al. (2013) showed the involvement of GPOX in the resistance of *Capsicum baccatum* var. *pendulum* to *Pepper yellow mosaic virus*. Siddique et al. (2014) reported that GPOX activity of two cotton (*G. hirsutum*) susceptible genotype (NIAB-111 and CIM-496) to CLCuBuV were reduced (26.49% and 31.54%, respectively) upon virus infection compared to healthy plants. Besides the antioxidant activity, peroxidases located in cell wall can also generate ROS (H_2O_2, OH^- ,

O^{2-}), which are toxic to pathogens, and participate in the strengthening of cell walls by lignification (Durner and Klessig 1997, Torres 2010). In our present study, high GPOX activity in MCPI coincided with lignin accumulation in cell walls compared with both CPU and CPI that showed lower GPOX activity (Fig. 8).

The APX activity was significantly reduced in MCPI plantlets compared to CPU and CPI (Fig. 6D). This finding is not in agreement with that of Kundu et al. (2013) who demonstrated a higher APX activity in the incompatible interaction between mungbean and MYMIV, at 7 DPI. However, as previously suggested by Durner and Klessig (1997), low CAT and APX activities are required to maintain higher H_2O_2 level. In MCPI, reduction in CAT and APX and increase in SOD and GPOX activities (Fig. 6) led to increased H_2O_2 level (Fig. 7A) and cell wall lignification (Fig. 8C). Contrarily, CPI plantlets in which well developed disease symptoms were observed, reduction in SOD and GPOX and increased CAT and APX activities (Fig. 5) with consequent decrease in H_2O_2 level (Fig. 7A) were observed and no lignin accumulation (Fig. 8B). In addition, Maia et al. (2013) reported the correlation between low APX activity with high H_2O_2 content, increase in GPOX and cell wall lignification in cowpea roots in response to salt stress.

Based on our results, we propose that the CAT, APX, SOD, and GPOX levels are important factors that determine the disease establishment or the resistance of cowpea to CPSMV infection (Fig. 9). The mutagenized CPSMV-resistant cowpea plants displayed coordinate and favorable alterations represented by increased SOD and GPOX activities associated with decreased CAT and APX activities in response to CPSMV-inoculation. These virus-induced activity modulation of these enzymes (Foyer and Noctor, 2005; Maia et al. 2013) provoked significant increases in the contents of H_2O_2 and phenolic compounds, which are associated with the oxidative burst, increased cell wall lignifications, and gene activation all representing an effective defense mechanism in the EMS-mutagenized MCPI which became resistant to CPSMV. For instance, this defense mechanism was also effective to protect the photochemical and CO_2 assimilation phases of photosynthesis as they were not affected by CPSMV inoculation in MCPI. Contrarily, in the susceptible cowpea, this defense mechanism did not operate and photosynthesis was strongly affected, which is probably associated with physiological disturbances induced by CPSMV-infection.

Fig. 9. A general scheme describing the main events that occur during cowpea–CPSMV interaction. (A) In CPI plantlets (susceptible) upon virus entry into the cytosol, ROS-generating processes are activated after plant-virus recognition. However, CPSMV counter-attacks by induction of CAT and APX activities leading to H_2O_2 scavenging to avoid toxicity favoring CPSMV replication and disease establishment. (B) In MCPI plantlets (mutagenized resistant) , higher SOD and GPOX activities and lower CAT and APX activities increase H_2O_2 levels leading to the oxidative stress in the cytosol and programmed cell death (PCD) at infection sites, which restrict CPSMV spreading. In addition, increased H_2O_2 levels can induce PAL and CHI activities to further hinder CPSMV infection. Red arrows indicate the cowpea processes induced or inhibited by CPSMV. Green arrows indicate the plant defense responses to CPSMV inoculation.



5. Conclusion

Seeds of the CE-31 cowpea genotype, highly susceptible to CPSMV, after EMS-treatment, generated resistant cowpea plantlets that apparently recognized virus elicitors and/or effectors and triggered biochemical defense responses that, in association with the maintenance of photosynthetic parameters, might confer resistance to CPSMV.

Author contributions

P. F. N Souza., F. D. A. Silva., I. M. Vasconcelos., and J. T. A. Oliveira., designed the study, wrote the manuscript (with input from all authors), performed all biochemical assays and interpreted the experimental data. J. A. G. Silveira., and F. E. L. Carvalho., produced and interpreted all physiological data and participated. All authors read and approved the final version of the manuscript.

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CAPÍTULO III

Comparative proteomic analysis of a susceptible cowpea (Vigna unguiculata [L.] Walp.) Genotype and its derived EMS-induced mutagenized resistant to *Cowpea severe mosaic virus*

PEDRO FILHO NORONHA DE SOUZA

Comparative proteomic analysis of a susceptible cowpea (Vigna unguiculata [L.] Walp.) Genotype and its derived EMS-induced mutagenized resistant to *Cowpea severe mosaic virus*

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Running title: **Proteomic analysis of a susceptible cowpea and its derived resistant mutagenized challenged with CPSMV**

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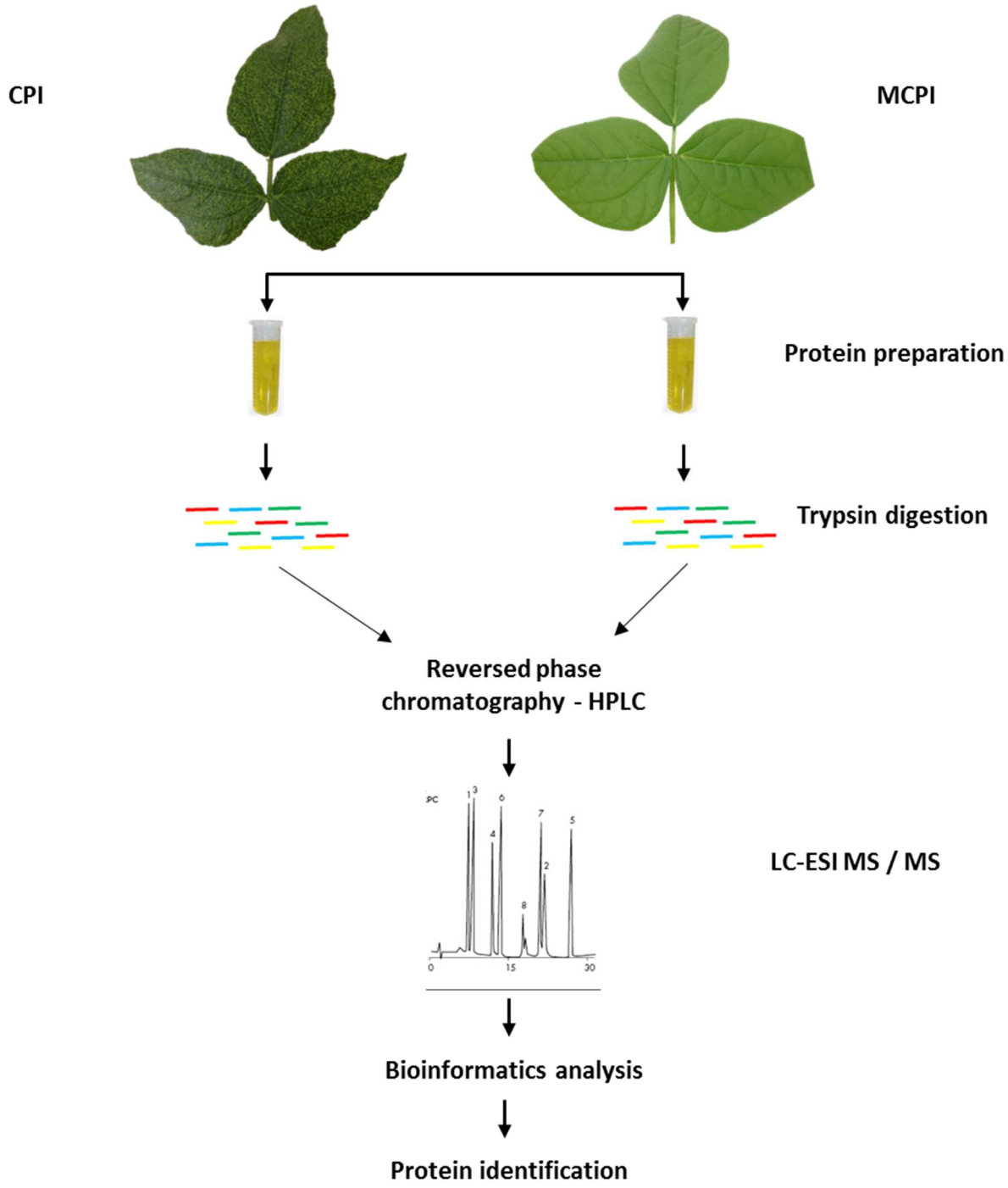
Highlights

• This is the first study to analyze proteomic changes in EMS treated cowpea plants challenged by CPSMV.
• 99 proteins showed differential accumulation after virus inoculation.
• We identified various host factors proteins that not described previously in cowpea-virus interaction.
• There are overlapping and specific proteomic responses in different cowpea plants.

Graphical Abstract



Abstract

Cowpea Severe Mosaic Virus (CPSMV) is one of the main constraints for cowpea (*Vigna unguiculata* L. Walp.) production due to the severity of symptoms, frequency of incidence, and the difficulties to control the pathogen. In the present work, the differential proteins accumulation of the susceptible cowpea genotype CE-31 and its devired mutagenized resistant plants obtained after the seed treatment with 0.04% ethyl methanesulfonate (EMS), both inoculated with CPSMV, was evaluated using label-free proteomics as an attempt to better understand the molecular basis of resistance (incompatible interaction) and susceptibility (compatible interaction) of cowpea to CPSMV. At 7 DPI with CPSMV, cowpea leaves were collected and the proteins extracted with Tris–phenol buffer, pH 8.0, digestion performed with trypsin, and the resulting peptides analyzed by LC-ESI MS/MS. Ninety nine proteins out of 249 identified were differentially represented. Regardless whether the susceptible genotype or the mutagenized resistant cowpea plantlets, CPSMV induce changes in proteome profile that involve several biological process, such as energy and metabolism, photosynthesis, response to stress, oxidative burst, and scavenging. Moreover, the CPSMV responsive proteins in the resistant mutagenized cowpea (MCPI) represent a complex network involving modulation of key cellular processes such as redox metabolism, photosynthesis, energy production and energetic metabolism, RNA interacting proteins, and protein metabolism, possibly relevant for resistance to CPSMV.

Biological relevance

Severe mosaic caused by CPSMV is one of the major diseases of cowpea crops. However, little is known about the defense mechanisms that cowpea employs to counterattack CPSMV. Therefore, we conducted a comparative proteomic analysis of a susceptible cowpea genotype and its derived EMS-induced mutagenized resistant to CPSMV as a strategy to assist in understanding the mechanisms involved in cowpea resistance. There are key differences in the abundance of proteins involved in the general metabolism of the resistant mutagenized. So that our comparative proteomic study hints at molecular mechanisms and pathways involved in the traits of susceptibility and resistance of cowpea to CPSMV.

Key words: *Vigna unguiculata*, EMS, CPSMV, Defense, Mass spectrometry

1. Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important legumes for the human consumption, particularly, in North and Northeast of Brazil. Cowpea seeds constitute a source of carbohydrates, vitamins and minerals, and possess good quality dietary proteins rich in lysine and tryptophan [1]. Cowpea is adapted to the semi-arid regions of the tropics and subtropics as it is tolerant within given limits to stresses like drought, high temperatures, poor soils, and salinity [2-4]. Unfortunately, cowpea is threatened by several diseases caused by pathogens, including severe mosaic, caused by CPSMV, which severely diminished productivity worldwide [5]. Currently, CPSMV control relies mainly on the use of resistant cultivars. Therefore understanding the defense mechanisms underlying cowpea resistance to CPSMV is crucial in plant breeding programs by the development of resistant plants.

CPSMV is a specie of the *Comovirus* genus, subfamily *Comovirinae*, family *Secoviridae* (International Committee on Taxonomy of Viruses, 2014 – ICTV, www.ictvonline.org/virustaxonomy.asp). This virus has a bipartite genome composed by two single stranded RNA molecules, positive-sense, denominated RNA1 and RNA2, both with a 5' linked protein VPg (Viral Genome-linked Protein), and a 3' polyadenylated tail. RNA 1 codes 5 proteins that functions in the replication of the viral RNAs and polyprotein processing: a proteinase cofactor (32 kDa); a helicase (58 kDa); VPg; a cystein proteinase (24 kDa); and a RNA-dependent RNA polymerase (87 kDa). RNA 2 codes a polyprotein that is cleaved to give a cell-to-cell and long distance movement protein, and the capsid (large and small) coat proteins [6-8].

During compatible host-virus interaction, biochemical responses like oxidative burst, which induces gene expression with subsequent pathogenesis-related proteins (PR-proteins) accumulation is weak or inexistent in the host plants. As a consequence, plants exhibit severe disease symptoms as mosaic formation, leaf deformation and necrosis that negatively affect photosynthesis and plant growth [9,10]. However, in incompatible interactions, plants recognize viral effectors that rapidly activate signaling cascades to induce defense mechanisms including alteration in Ca^{2+} influx, ROS generation, particularly H_2O_2, in the chloroplast and mitochondrion followed by programmed cell death (PCD), which characterizes the hypersensitive response (HR),

and systemic acquired response (SAR) with accumulation of pathogenesis-related proteins (PR-proteins) to avoid virus spreading to healthy tissues, preventing local and systemic disease establishment [11-13].

Ethyl Methanesulfonate (EMS) is a chemical mutagenic agent ($C_3H_8SO_3$) that induces random point mutations by replacement of a single base nucleotide with another nucleotide of the genetic material. However, such mutation preferentially modifies guanine residues to *O*-6-ethylguanine, an abnormal base that is added naturally to DNA molecules by DNA polymerase [14] so that during DNA replication thymine instead of cytosine is placed opposite to *O*-6-ethylguanine. Such alteration can lead to the substitution of the original G:C base pair by A:T pair (transition mutation) after successive replication steps [15]. Several works report that changes in plants caused by EMS treatment resulted in resistance to pathogens, including virus [16-17]. For example, EMS-treated (0.002% v/v) *Arabidopsis thaliana* seeds that were susceptible to TuMV (*Turnip Mosaic Virus*, genus *Potyviruses*) produced plants that became resistant. This alteration was termed “loss of susceptibility” [17].

This current study is the first report on the comparative proteomic analysis using label-free protein quantification method with LC-ESI MS/MS coupled with database searching to identify and compare changes in the relative abundance of proteins in a susceptible cowpea (*Vigna unguiculata* [L.] Walp.) genotype and its derived EMS-induced resistant mutagenized, both challenged with CPSMV, hoping that it could shed light on the mechanisms of resistance and susceptibility to this virus.

2. Materials

2.1 Cowpea plants

Cowpea seeds, genotype CE-31 (syn. Pitiuba), highly susceptible to CPSMV, were obtained from the Department of Biochemistry and Molecular Biology of Federal University of Ceara, Brazil. Seed surface sterilization was carried out by immersion in 1% (v/v) sodium hypochlorite (0.05% of active chlorine) for 3 min, followed by exhaustive (10x) washed with sterile ultra-pure water. To break dormancy, the seeds were immersed in sterile ultra-pure water for 20 min, at room temperature (23 ± 2 ºC).

To obtain the cowpea mutagenized plantlets, cowpea seeds, genotype CE-31, were treated with 0.04% (v/v) EMS for 20 h, instead of 0.002% (v/v) for 12 h as in [17], and washed exhaustively (30x) with sterile ultra-pure water. Next, seeds were planted in filter papers (Germitest^{TM}, 28 x 38 cm) in the dark and watered twice a day with sterile ultra-pure water. Three days after planting, germinated seeds were selected and transferred to 1.5 L plastic pots (five per pots) containing river sand previously washed with tap water (7x) followed by distilled water (3x), and autoclaved (121 °C, 30 min, 1.5 × 10^5 Pa). The transplanted plantlets were maintained in greenhouse conditions, exposed to 12-h natural light with the photosynthetic photon flux density (PPFD) varying from 300-650 µmoles m^{-2} s^{-1} (190SA quantum sensor, LI-COR, USA), temperatures of 31.0 ± 3.0 °C (day) and 27.0 ± 0.8 °C (night), and 75.8 ± 8.9% relative humidity. Watering of cowpea plants was done daily with distilled water for up to 3 day after transplantation to the jars followed by irrigation with 100 mL nutritive solution [18] as previously modified [2].

2.2 Virus inoculums

Cowpea severe mosaic virus was obtained from leaves of the cowpea genotype CE-31 infected with the CPSMV isolate from Ceara state, Brazil [19] (thereafter referred as CPSMV), showing typical symptoms of severe mosaic, maintained in a greenhouse at the Department of Biochemistry and Molecular Biology, Federal University of Ceara, Brazil. To prepare the inoculums the CPSMV infected cowpea leaves were macerated with 10 mM K^+-phosphate buffer, pH 7.0, containing 0.1% (m/v) sodium sulfite and to this suspension the abrasive carborundum (500-600 mesh) was added (1:10, m/v) [20]. Health fully expanded cowpea secondary leaves from 15 day-old plantlets of CE-31 genotype were manually infected with CPSMV by rubbing the CPSMV inoculum on the adaxial and abaxial leaf surfaces using the tips of the index finger and thumb with hands protected with surgical gloves. Two groups of cowpea plantlets were used in the experiments: (a) plantlets grown from CE-31 original seeds and inoculated with CPSMV, designated “CPI” group; (b) plantlets grown from the CE-31 seeds that were previously treated with 0.04% (v/v) EMS for 20 h and inoculated with CPSMV, designated “MCPI” group. MCPI represented mutagenized plants that became resistant to CPSMV without disease symptoms (**Table 1**). Plants were arranged in a completely randomized block design with 3

biological replicates per block (pot). The physiological measurements were done in the entire plants at seven days post inoculation (7 DPI) from 9:00 to 11:00 AM before collection for proteomics analysis. For the proteomics analyses, the leaves of CPI and MCPI groups were detached soon after the physiological measurements (7 DPI) and stored at -80 °C until used. The presence of hydrogen peroxide (H_2O_2) and programmed cell death evaluation were done in secondary fresh leaves collected from CPI and MCPI groups at 7 DPI, which were immediately used for analysis.

2.3 Protein Extraction

Protein extraction from cowpea leaves was performed as previously described by Yao et al. [21] and slighted modified by Moura et al. [22]. CPSMV-inoculated cowpea secondary leaves (2 g) were ground in liquid nitrogen to obtain a fine powder and 15 mL of a solution containing 10% (m/v) trichloroacetic acid (TCA) and 2% (v/v) 2-Mercaptoethanol (2-ME) in acetone were added. The suspension was vortex-mixed for 60 min, centrifuged (High-Speed Refrigerated Centrifuge – HITACHI, Tokyo, Japan, 18,000 × *g*, 15 min, 4 °C), and the supernatant discarded. The resulting precipitate was washed three times with cold 2% (v/v) 2-ME in acetone, vortex-stirring for 10 min, and centrifuged as above. The precipitate was collected, and dried in a hermetically-sealed chamber containing silica particles, at 4 °C, overnight, and solubilized with 6 mL of 0.1 M Tris–HCl, pH 8, buffer, containing 30% (m/v) sucrose, 2% (m/v) SDS, 10^{-3} M PMSF, and 10^{-3} M PVPP (Tris-8 buffer), at 4 °C, for 10 min. After centrifugation at 10,000 × *g*, 4 °C, 10 min, an equal volume of Tris–phenol pH 8.0 (Sigma) was added to the resulting supernatant, and the mixture centrifuged at 18,000 × *g*, 15 min, 4 °C. The upper phenol phase formed was collected, 4 volumes of ice-cold 0.1 M ammonium acetate/methanol solution added, the mixture incubated for 120 min, at −80 °C, and centrifuged at 18,000 × *g*, 15 min, 4 °C. The resulting precipitate was ressuspended and incubated for 20 min, 4 °C, with ice-cold 80% (v/v) acetone, followed by centrifugation at 10,000 x *g*, 10 min, 4 °C. This washing procedure was repeated four times at the end of which the samples were air-dried in the presence of silica particles, at 4 °C, overnight. The protein concentration was measured [23] using a standard curve prepared with known concentration of bovine serum albumin (BSA). The protein samples (leaf extracts) recovered were stored at −20 °C until used for proteome analysis.

Table 1. Control (CPU) and experimental (CPI, MCPI) plant groups

Plant groups	0.04% EMS-treated	CPSMV-infected	CPSMV-free buffer	Disease symptoms
CPI[a]	No	Yes	No	Yes
MCPI[b]	Yes	Yes	No	No

[a] Plants originated from cowpea (CE-31 genotype) seeds not previously treated with 0.04% (v/v) EMS, but inoculated with CPSMV.

[b] Plants originated from cowpea (CE-31 genotype) seeds previously treated with 0.04% (v/v) EMS, and inoculated with CPSMV.

2.4 Protein Digestion

To resuspend the protein pellets for trypsin digestion, 20 µL of 0.05 M ammonium bicarbonate (Sigma) and 50 µL of RapiGest SF (1 mg/mL - Waters) were added, the mixture incubated for 15 min at 80 °C under vortex-stirring, and centrifuged at 10,000 x *g*, for 15 s, at room temperature (23 ± 2 °C). Next, 5 µL of 0.1 M dithiothreitol (GE Healthcare) were added and the samples stirred and incubated at 60 °C for 30 min. After incubation, the samples were left to cool at room temperature (23 ± 2 °C) and centrifuged at 10,000 x *g*, for 15 s. Subsequently, 5 µL of 0.3 M iodoacetamide (GE Healthcare) were added to the sample supernatants, which were incubated for 30 min at room temperature, protected from light. Then 20 µL of a trypsin solution (Promega) in 0.05 M ammonium bicarbonate (Sigma) were added to the samples (1:100, enzyme/sample), and incubated at 37 °C for 15 h to allow digestion. At the end of the process the samples were centrifuged at 22,000 x *g* for 30 min, at 6 °C, the supernatants collected in a microcentrifuge tube (1.5 mL) and evaporated in a vacuum concentrator (Labconco). Lastly, the samples were ressuspended with 0.1% (v/v) formic acid in 50% (v/v) acetonitrile, centrifuged at 21,000 x *g* for 5 min at 23 °C and the final supernatants containing the tryptic peptides recovery for posterior analysis.

2.5 LC-ESI-MS/MS Analysis

Previous chromatographic separation of the samples containing the tryptic peptides was done on a XR-ODS C18 column (2.0 mm x 30 mm; 2.2 microns; Kyoto, Japan) coupled to a HPLC system (Shimadzu - Kyoto, Japan), joined to a micro TOF ESI-Q III mass spectrometer (Bruker Daltonics, Germany) using ESI in positive linear mode. The mobile phases were 0.1% (v/v) formic acid in 2% (v/v) acetonitrile (solvent A) followed by 0.1% (v/v) formic acid in 80% (v/v) acetonitrile (solvent B). The gradient used was: 5% (v/v) solvent B for 5 min; 5-50% (v/v) solvent B for 50 min; 50-95% (v/v) solvent B for 10 min; 95% (v/v) solvent B for 8 min; 95-5% (v/v) solvent B for 1 min; and 5% (v/v) solvent B for 6 min, with a flow rate of 0.4 mL min^{-1}. Other parameters were: spray voltage, 4.5 kV, using nitrogen (N_2) as a source of ionization; nebulizer 4.0 Bar; 8.0 dry gas; dry temperature 200 °C. Ions were scanned in a range of 300-3000 m/z followed by five MS/MS scans. Three independent runs were performed for

each sample. Analysis of mass fragmentation spectra (MS/MS) was done using the Data Analysis software version 4.0 (Bruker Daltonics, Germany). Spectra were deconvoluted using a deconvolute tool to determine peptides charge. These data were loaded on the MASCOT algorithm (Matrix Science, London, United Kingdom, version 2.3) and in order to identify proteins in databases they were loaded to the Biotools program (Bruker Daltonics, Germany). Searches were performed using databases for *Glycine max* (taxid: 3847)*, Phaseolus vulgaris* (taxid: 3885)*, Vigna unguiculata* (taxid: 3917) and *Fabales* (taxid: 72025) all downloaded from NCBI database. In addition, the VigGS database (January 25, 2016) [24] was searched to validate protein identification using the above databases. The parameters used in the searches were: trypsin selected as the proteolytic enzyme; carbamidomethylation of cysteine as fixed modification; methionine oxidation as variable modification. Precursor ion mass tolerance (MS) and fragment ion mass (MS/MS) tolerance of ± 0.2 Da was used. Proteins were considered identified when the corresponding ion peptide score had confidence interval greater than 99.5% confidence ($p < 0.05$) taking into consideration the MASCOT score.

2.6 Protein Quantification

Quantification of proteins present in the two studied plantlet groups, and that appeared in all biological triplicates analyzed, was done using the Data Analysis tool version 4.0. Three ions for each peptide associated with an identified protein were selected using the Extracted Ion Chromatogram (EIC) tool. The peak area of every ion was calculated with the Integrate Chromatogram (IC) tool, and the data used for comparison between the plantlet groups (MCPI vs CPI). Only exclusive leaf proteins from both MCPI and CPI, and overlapping that showed at least 2-fold up-accumulation (≥ 2.0) and decline (≤ 0.6) in concentration, with $p < 0.05$, in relation to CPI, were taken into consideration for comparison.

2.7 Gene ontology, subcellular location, and protein-protein interaction network

Gene ontology (GO) annotation and prediction of subcellular location of proteins were carried out using a free access database (http://www.uniprot.org/) and the free program Plant-mPLoc version 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/), respectively. The differentially up- and down-represented cowpea proteins were blasted against *A. thaliana* in STRING version 10.0 database (http://string-db.org/) to constructed the protein-protein interaction network.

2.8 H_2O_2 analysis and cell viability

Light microscopy visualization of H_2O_2 in fresh cowpea leaves from CPI and MCPI was done after Thordal-Christensen *et al*. [25]. Briefly, the cut end of petioles of detached cowpea leaves were immersed in a solution containing 1.0 mg mL^{-1} DAB (3´-3´-diaminobenzidine, Sigma) in the dark at room temperature (23 ± 2 °C), for 8 h. Then the leaves were decolorized by incubation, for 48 h, in a bleaching solution (three changes) composed of 1.5 g L^{-1} TCA in a 3:1 (v/v) mixture of ethanol-chloroform. To prepare the DAB solution (1.0 mg. mL^{-1}), the reagent was mixed with ultra-pure water, the pH adjusted to 3.0 with 1.0 M HCl, and the solution heated to 50 °C for 1 h to dissolve the powder. Next, the pH was adjusted to 4.0 with 1.0 M NaOH and the final volume completed with ultra-pure water. For light microscopy (Olympus System Model BX60F5 microscope) visualization, leaf pieces were mounted with 50% glycerol on glass slides. Analysis was performed in three biological replicates.

Cell viability in fresh cowpea leaves from CPI and MCPI at 7 DPI was evaluated using Trypan Blue-lactophenol as a vital stain [26]. Cowpea leaves were decolorized by incubation overnight in the bleaching solution previously described and stained by incubation with lactophenol-trypan blue solution (0.01% [v/v] trypan blue in lactic acid:phenol:ultrapure water, 1:1:1[v/v/v]) overnight. Excess wet stain solution was removed using ultra-pure water. Non-viable cells absorb the dye and appeared as blue spots. Intact cells remained unstained.

2.9 Gas exchange and chlorophyll fluorescence measurements

Net of CO_2 assimilation (Pn), stomatal conductance (gs) intercellular CO_2 partial pressure (Ci), and photochemical activity were measured using a portable Infrared Gas Analyzer System (IRGA), equipped with a leaf chamber fluorometer (LI-6400-40, LI-COR, Lincoln, NE, USA). Conditions of measurements were: PPFD of 1000 μmol m^{-2} s^{-1}, 28 °C, air vapor pressure deficit of 1.0 ± 0.2 kPa, and air CO_2 partial pressure of 38 Pa. The instantaneous carboxylation efficiency was calculated as PN/Ci. The fluorescence parameters were measured using the saturation pulse method in both light and dark-adapted (30 min) leaves. The intensity and duration of the light saturation pulse were 8000 μmol m^{-2} s^{-1} and 0.7 s, respectively. The amount of blue light was set to be 10% of the PPFD to maximize stomatal aperture [27]. The following photochemical parameters were assessed: the maximum quantum efficiency of PSII [Fv/Fm = (Fm − Fo)/Fm], the effective quantum efficiency of PSII [ΔF/Fm′ = (Fm′ − Fs)/Fm′]. The apparent electron transport rate [ETR = (ΔF/Fm′ × PPFD × 0.4 × 0.84)], where 0.4 was used as the fraction of excitation energy distributed to PSII, and 0.84 was used as the fraction of incoming light absorbed by the leaves. The non-photochemical quenching coefficient [NPQ = (Fm − Fm′)/Fm′]. The Fm and Fo are the maximum and minimum fluorescence of dark-adapted leaves, respectively; Fm′, Fo´ and Fs are the maximum, minimum and steady-state fluorescence in the light-adapted leaves, respectively [28].

2.10 Determination of chlorophyll and carotenoid content

Total Chlorophyll (Chl *t*), Chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoids were performed spectrophotometrically as described by Lichtenthales and Welburn [29]. 0.2 g of frozen leaves extracted with 85% (v/v) aqueous acetone and centrifuged at 4000-x g for 10 min 4 °C. The supernatant was measured at three wavelengths of 470 nm for carotenoids and 646 and 664 nm for Chl *t*, Chl *a* and *b* in Genesys 10S UV-vis spectrophotometer (Thermo Scientific, USA).

2.11 Virus detection and gene expression validation

2.11.1 PCR primer design

Virus detection in cowpea leaves was done by semi-quantitative RT-PCR using degenerated primers (Forward: 5'-GCATGGTCCACWCAGGT-3'; Reverse: 5'-YTCRAAWCCVYTRTTXGGMCCACA-3'), design to align with the sequence of the viral RNA that encodes the coat protein, as previously described Brioso et al. [30]. Primers used in the RT-qPCR reaction for catalase-2 (CAT-2) and 2-cys-peroxiredoxin 2a (2CysprxA) were designed based on *V*. *unguiculata* expressed sequence tags (ESTs) obtained from blast searches (Table 2) against EST database (NCBI - http://www.ncbi.nlm.nih.gov/). Five housekeeping genes (phospholipase A2 [*PP2A*], 60S ribosomal protein L23a [*L23a1* and *L23a3*], insulinase [*Insu*], and pha-F-box protein [*phaFbox*]) were used to obtain the most stable reference genes to be used for normalization of the RT-qPCR experiments. The Perl Primer v1.1.19 software [31] was used to design the primers with the following characteristics: melting temperature (Tm) between 57 and 64 °C; length of 18–24 bp, and amplicon size of 103 to 162 bp (Table 2).

2.11.2 RNA extraction and cDNA synthesis

Total RNA was extracted from approximately 0.1 g of cowpea leaves from CPI and MCPI plantlet groups, after they have been pulverized in liquid nitrogen, using the NucleoSpin® RNA Plant (Macherey Nagel, Germany) kit according to the manufacturer's instructions. During RNA purification process, DNase was used to eliminate contamination with genomic DNA. The amount of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) at 260 nm and RNA integrity analyzed (0.5 µg of RNA) by electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.5 µg mL^{-1}). The cDNA Synthesis was carried out using the ImProm-II™ Reverse Transcription System according to the manufacturer's instructions. The cDNA obtained was stored at −20◦C until used.

2.11.3 Virus detection

The cDNA fragment corresponding to the viral coat protein of CPSMV was amplified by semi-quantitative RT-PCR using the following parameters: an initial denaturation step of 95 °C for 5 min, 40 cycles each consisting of the denaturation step in which the reaction mixture was heated at 94 °C for 20 s, annealing step at 41 °C for 20 s, and the extension step at 72 °C for 45 s. In the last cycle, the extension step was done at 72 °C for 10 min. Upon completion of the reaction, the products were visualized (ethidium bromide-staining) after 2% (m/v) agarose gel electrophoresis run carried out in a Pharmacia Biotec electrophoresis unit for 40 min, at 25 °C, 100 V, 50 mA.

2.11.4 Gene expression and data analysis

RT-qPCR reactions were performed using the Mastercycler® ep realplex (Eppendorf AG, Hamburg) system. Assays were conducted in 96 wells plates as indicated by the GoTaq® qPCR Master Mix (Promega) kit. Reaction consisted of 1 µL of each primer (300 nmoles), 4 µL of cDNA (25 µg), 10 µL 1x GoTaq® qPCR Master Mix and 4 µL of ultrapure water in a final volume reaction of 20 µL. The same aliquots of cDNA sample were used in all sets of experiment. Optimal annealing temperatures for each primer pair were obtained using a temperature gradient reaction from 47 to 65 °C (Table 2). The PCR assays were performed in triplicate and the reactions run using the following parameters: 2 min at 95 °C for activation of the enzyme, followed by 40 cycles each consisting of denaturation at 95 °C for 15 s, annealing at a temperature range of 57.5 °C to 63.6 °C (Table 2) for 20 s, extension at 60 °C for 20 s, and the last cycle of final extension step at 72 °C, 10 min. After amplification, the melting curves were constructed by holding temperature at 95 °C for 15 s, 60 °C for 15 s, followed by heating slowly to 95 °C at 0.03 °C s^{-1}. RT-qPCR reactions were performed in triplicate and *Ct* values were averaged. Primer efficiency was determined diluting the cDNA samples towards obtaining 80-100% efficiency. Melting curves were analyzed to verify the absence of unspecific products and/or primer/dimer formation. Gene normalization was performed using two reference genes (*phaFbox and L23a3*) out of 5 tested and validated using the genorm^{PLUS} module in qbase^{PLUS} version 1.5

(Biogazelle) [32,33]. Evaluation of relative change in gene expression was carried out using the $2^{-\Delta\Delta CT}$method [34].

3. Results

3.1 CPSMV interaction with cowpea

CPSMV failed to induce the characteristic mosaic symptoms in the mutagenized resistant cowpea plantlet group (MCPI), at 7 DPI, indicating that the previous treatment of the CE-31 seeds with EMS change the phenotype of susceptibility to resistance even after 3 months CPSMV infection (**Fig. 1A and C**). Indeed the CPI group, constituted of plantlets of the high susceptible genotype CE-31, showed all the characteristic symptoms of severe mosaic at 7 DPI: abnormal leaf morphology and the presence of yellow patches, mosaic, and chlorosis (**Fig. 1B**). This symptoms were compatible with the detection of the viral particles by RT-PCR in CE-31 infected plantlets (CPI group) at 7 DPI (**Fig. 1D Lane – 2**), absent in the cowpea mutagenized plantlets (MCPI group) that were equally infected with CPSMV (**Fig. 1D Lane – 1**).

3.2 Identification and biological classification of the differentially represented proteins of cowpea challenged with CPSMV

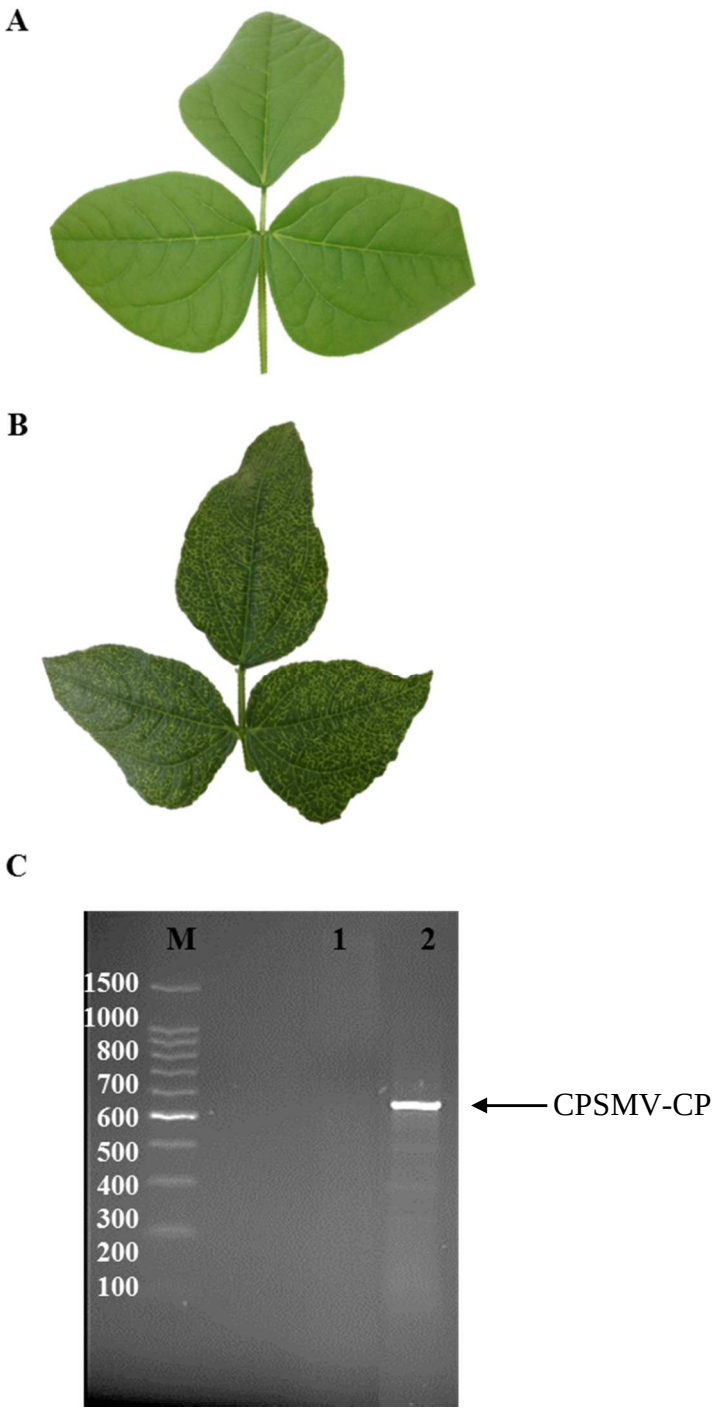
In this current study, changes in the proteome profiles of the secondary leaves of MCPI and CPI cowpea plantlets (**Table 1**) submitted to artificial inoculation with CPSMV were investigated at 7 DPI. After LC-ESI-MS/MS analysis of the tryptic peptides from both experimental groups and search against the non-redundant NCBI and Uniprot database using the Mascot daemon program (http://www.matrixscience.com/search_form_select.html), 249 proteins were identified, 141 belonging to MCPI and 108 to CPI (data not shown).

Table 2

Primer sequences of reference and target genes used for gene expression analysis

Gene	Primer sequence (5'-3')	Amplicon (pb)	Accesion Number	Optimal Melting Temperature (Tm °C)
Cat2	F 5' GTTCCACAAGATTACAGGCAT 3' R 5' TCTTCCTCCAATAGACACTTCA 3'	134	FJ392011	57.5
2CysPrxA	F 5' AATTGCTCATCTGTATCAGTC 3' R 5' GTTCCATACATCATAAACCGT 3'	162	JF438998.1	57.5
PP2a2	F 5' GTCGCTGTGTAGGATTGGAG 3' R 5' AAGAAGAAACTCGTGCTGTG 3'	130	XM_014658159	57.5
L23a1	F 5' GCTGTTTCAACCCTAAACCCT 3' R 5' AGAAGAAGATCAAGGACGCA 3'	103	XM_014641092.1	57.5
L23a3	F 5' CAGGGCATCATAGTCAGGT 3' R 5' AGGCTTAACATCGAGTAGG 3'	125	XM_014641511.1	57.5
Insu	F 5' GCAACCAACCTTTCATCAG 3' R 5' TCCTTTGCTCAATGTTCCC 3'	143	FG9062104.1	57.5
F-Box	F 5' GCTTATTCAATCCGCTTGTC 3' R 5' GTCCTATAACAGCTTCTCCA 3'	148	FG812521.1	57.5

Figure 1. Mutagenized resistant (MCPI) (**A**) and susceptible (CPI) (**B**) cowpea plants 7 days after inoculation with CPSMV. In **A**, MCPI leaves inoculated with CPSMV that not have disease symptoms. In **B**, CPI leaves inoculated with CPSMV showing severe symptoms of disease. (**C**) Agarose gel electrophoresis (1.5%) of the amplified cDNA fragment of the CP-CPSMV; **Lane M =** Molecular Weight markers; **Lane 1** = the amplified cDNA fragment of CP-CPSMV extracted from the infected cowpea leaves of MCPI plants; **Lane 2** = the amplified cDNA fragment of CP-CPSMV extracted from infected CPI plants.



Comparative analysis between the MS/MS area peaks of the identified proteins from MCPI and CPI using the Data Analysis Software version 4.0.1 (Buker, Daltonics) showed 99 differentially represented proteins at 7 DPI (**Table 3**): 52 proteins only detected in MCPI; 24 proteins only detected in CPI; and 23 proteins present in both studied groups. Moreover, taken into consideration at least two-fold change ($p < 0.05$), 16 proteins over-accumulated and 7 decreased their level in MCPI when compared to CPI (**Fig. 2, and Table S1**).

GO annotation for biological functions allowed to classify these 99 identified proteins into 14 categories: Amino acid Metabolism (6); Carbohydrate binding and Signaling (3); Cell Redox Homeostasis (11); Cell Structure (3); Strees and Defense Response (15); Energy and Metabolism (13); Isoflavone Biosynthesis (2); Nucleotide Binding (6); Photosynthesis and Photorespiration (23); Protein Metabolism (5); Regulation Factors or RNA binding (7); Transferase (3); and Unknown (1) (Fig. 3A). In regarding to the subcellular localization, most of the 99 identified proteins are from Chloroplast (46) and Cytoplasm (22). The remaining are from Cytoplasmic/Cell Membrane (1), Cytoplasmic/Nucleus (3), Cytoplasmic/Secreted (2), Endoplasmic Reticulum/Golgi apparatus (2), Endoplasmic Reticulum/Mitochondrion (2), Golgi apparatus (2), Lysosomes (1), Mitochondrion (5), Nucleus (6), Peroxisome (5) Tonoplast (1), and Unknown (1) (**Fig. 3B**).

Importantly, several proteins included in the class of plant stress and defense response were found to be over-represented at least two-fold or were detected only in MCPI, in comparison to CPI, such as Glycine-rich RNA-binding protein-7 like, Glycine-rich RNA-binding protein, cysteine and serine proteinase inhibitors, and Cysteine proteinase RD21a (**Table 3**). Two protein kinases, CBL-interacting protein kinase (CBL-CIPK) and L-type Lectin-domain containing receptor kinase (LRK), included in the class of carbohydrate binding and signaling, were detected only in MCPI. Moreover, the MCPI plantlet group showed increased abundance of cell redox homeostasis-related proteins such as two CuZn-superoxide dismutase (CuZn-SOD, located in the chloroplast and cytosol, respectively), and a peroxisomal glycolate oxidase (GOX), which are enzymes that increase H_2O_2 levels. In CPI, an isoform of catalase (CAT-4) and an ascorbate peroxidase (APX), both involved with H_2O_2 scavenging, increased in abundance, but not enzymes that produce H_2O_2, like SOD (**Table 3**). These findings related to the increased abundance of CuZn-SOD and GOX in MCPI are coincident with the increased H_2O_2 level (**Fig. 4**) and PCD events (**Fig. 5**)

verified for this plantlet group in comparison to the CPI plantlets. In parallel, MCPI plantlets showed increased abundance of enzymes that scavenge H_2O_2, such as Catalase 2 (CAT-2), 2-Cys peroxiredoxin (2-CysPrxA), Peroxiredoxin-2E (Prx2E), and Thioredoxin M-type (TrxM) (**Table 3**). Unsurprisingly, over-accumulation of CAT-2 and 2CysPrxA in MCPI compared to CPI plants was confirmed by qRT-PCR (**Fig. 6**). Thus, it is likely that the coordinated accumulation and scavenging of ROS promoted by these enzymes in the immune plants of the MCPI group are crucial for the H_2O_2 homeostasis favoring cowpea defense against CPSMV.

To cope with their natural enemies plants require resources and energy. Interestingly, all identified proteins classified as related to photosynthesis and photorespiration over-accumulated in MCPI plantlets when compared to CPI (**Fig. 3A**). These proteins are oxygen-evolving enhancer protein, photosystem I subunit PsaD, Ribulose bisphosphate carboxylase, Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, Chlorophyll A/B binding protein 8, photosystem II D1 (PSIID1), and photosystem II D2 (PSIID2) (**Table 3**).

On the other hand, the CPI plantlets showed increased abundance of proteins involved with protein metabolism such as Ribosomal protein S8, Ribosome recycling factor, and 40S ribosomal protein S20-1-like. In addition, in CPI two isoforms of actin involved in cell structure, eEF-1A and eEF-1B involved in RNA metabolisms, DEAD-box ATP-dependent RNA helicase 9-like (DDX), three proteins of the 70 kDa heat shock family, and two proteins of the 90 kDa heat-shock family were all over-accumulated in relation to those of MCPI.

Figure 2. Venn diagram of differentially represented proteins identified in MCPI and CPI cowpea plants challenged with CPSMV.

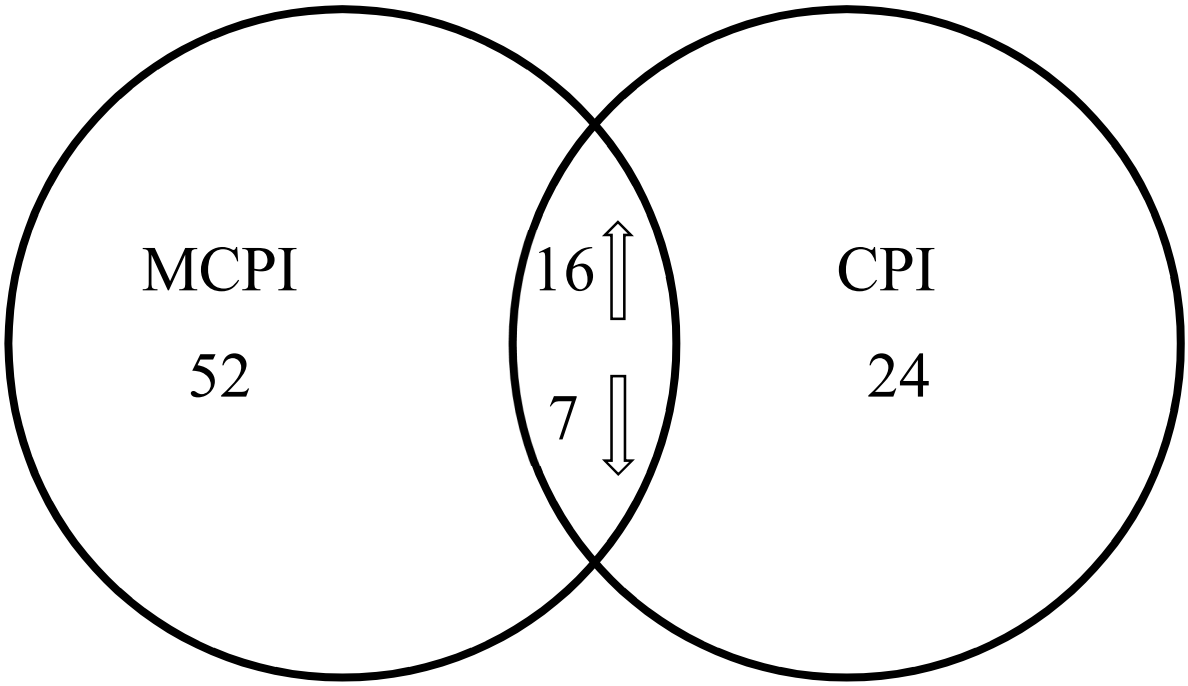


Figure 3. Representation of differentially accumulated proteins for Biological processes **(A)** according with Gene Ontology (GO) classification annotation and Subcellular location **(B)** according Plant-mPLoc free online server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/), highlighting differential distribution in CPI and MCPI plants at 7 dpi with CPSMV. White and black bars represent proteins are up- or down-accumulated protein, respectively.

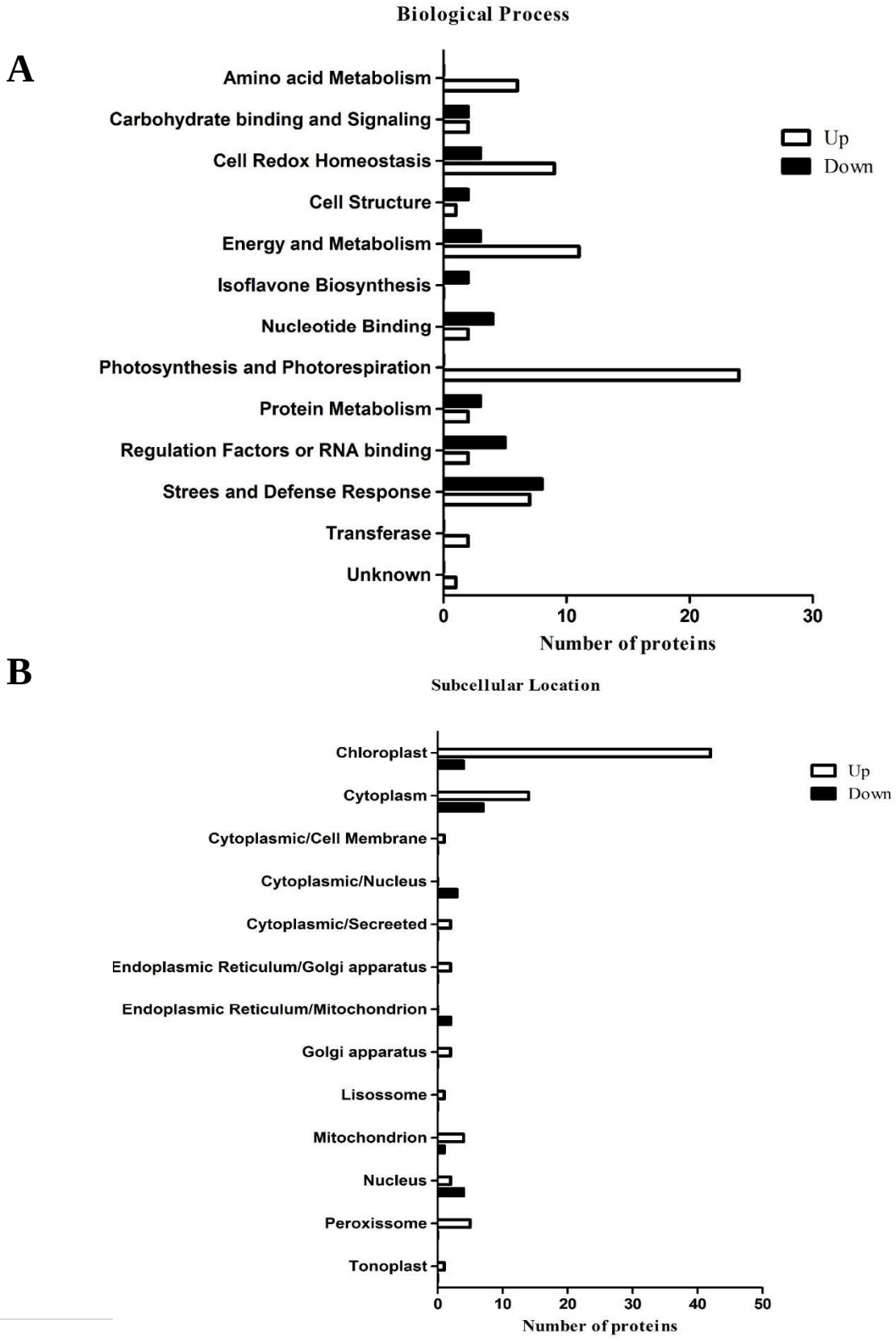


Figure 4. Microscopic analysis of H_2O_2 accumulation in the primary leaves of the (**A**) CPI and (**B**) MCPI cowpea plants (*V. unguiculata*) genotype CE-31 at 7 days post inoculation (dpi) with CPSMV. The leaves were stained with 3´diaminobenzidine (DAB). This result were obtained from three biological replicates.

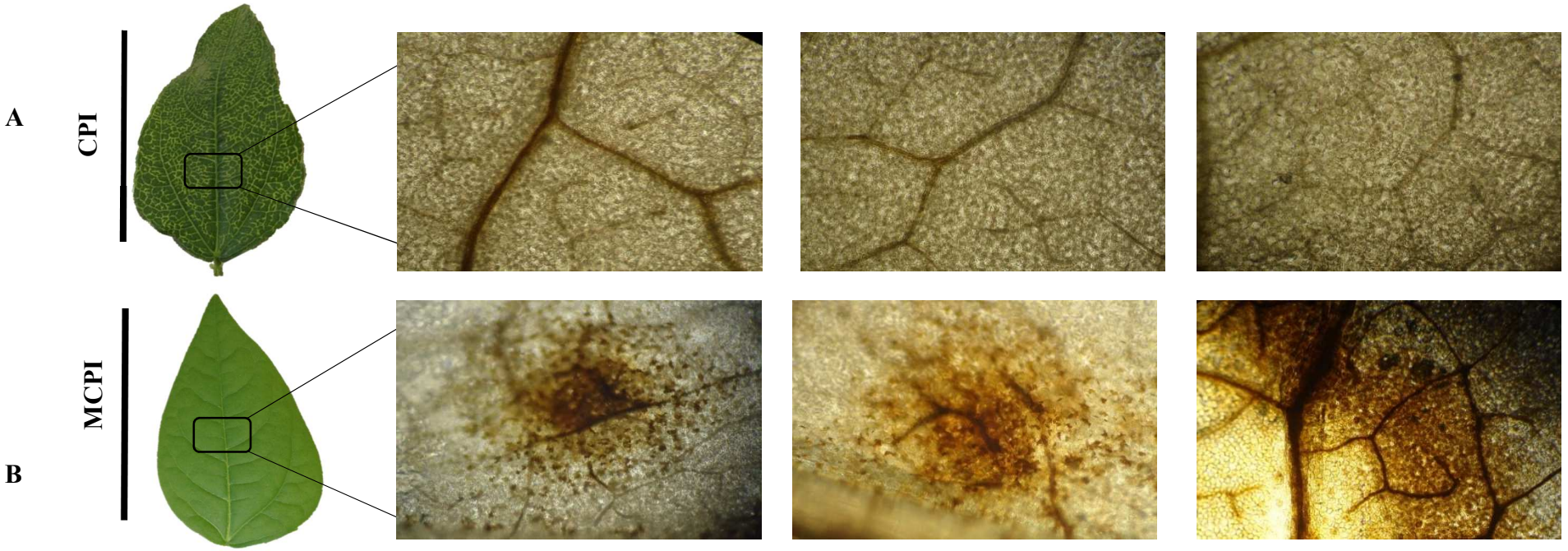


Figure 5. Cell viability assay using Trypan blue staining of leaves. (**A**) Leaves of CPI and (**B**) MCPI cowpea plants at 7 dpi with CPSMV.

Figure 6. Relative gene expression of 2CysPrxA (**A**) and CAT2 (**B**) in leaves of cowpea 7 days post inoculation (DPI) with CPSMV. Results are means ± standard deviation of three qRT-PCR reactions.

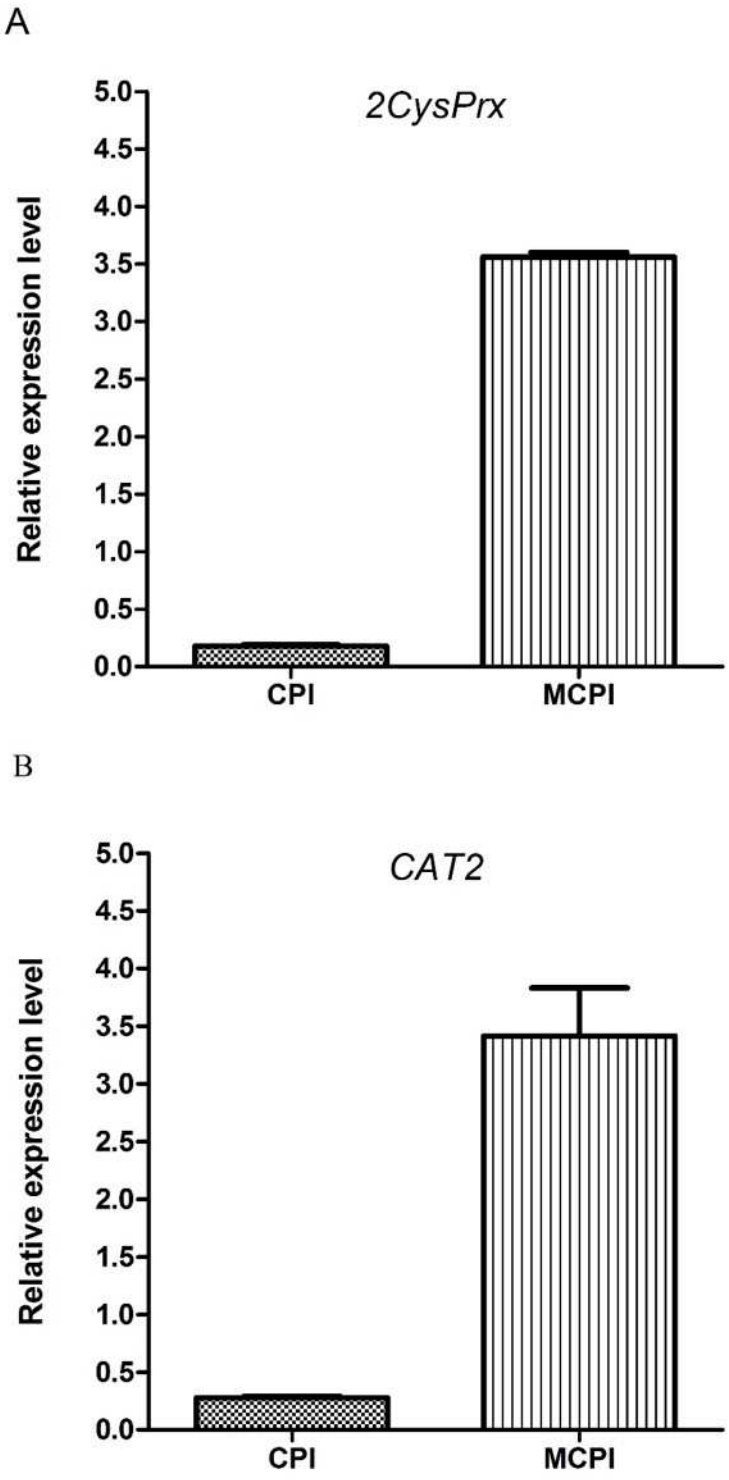


Table 3

Differentially expressed proteins identified by ESI-LC-MS/MS.

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
Amino Acid Metabolism				
Glutamine synthetase precursor	Vigan.05G076400.01	*Vigna radiata*	Chloroplast	Unique to MPCI
Serine hydroxymethyltransferase isoform X1	gi|356552984	*Glycine max*	Mitochondrion	Unique to MPCI
Serine hydroxymethyltransferase	Vigan.01G291600.01	*Vigna radiata*	Mitochondrion	Unique to MPCI
Cysteine synthase	gi|148562457	*Glycine max*	Mitochondrion	Unique to MPCI
Delta 1-pyrroline-5-carboxylatesynthetase	gi|13161405	*Vigna unguiculata*	Cytoplasm	Unique to MPCI
Glutamine synthetase partial	Vigan.05G076400.01	*Vigna radiata*	Mitochondrion	Unique to MPCI
Carbohydrate binding and Signaling				
CBL-interacting protein kinase	gi|154425355	*Vigna unguiculata*	Cell Membrane/Cytoplasm	Unique to MPCI
L-type lectin-domain containing receptor kinase	gi|356547001	*Glycine max*	Cytoplasm	Unique to MPCI
Lectin precursor	gi|41059971	*Vigna unguiculata*	Cytoplasm	Unique to CPI

Table 3 (continued)

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
Cell Redox Homeostasis				
Copper/zinc superoxide dismutase	gi|558695578	*Phaseolus vulgaris*	Chloroplast	5.871
2-Cys peroxiredoxin BAS1-like	Vigan.04G351300.01	*Vigna radiata*	Chloroplast	2.618
Peroxiredoxin-2E	gi|558695536	*Phaseolus vulgaris*	Chloroplast	2.72
Catalase, 2 partial	Vigan.02G020900.01	*Vigna radiata*	Peroxisome	Unique to MCPI
PREDICTED: peroxiredoxin-2E	gi|356572518	*Glycine max*	Chloroplast	Unique to MCPI
Peroxisomal glycolate oxidase	gi|543176674	*Phaseolus vulgaris*	Peroxisome	Unique to MCPI
CuZn-superoxide dismutase	Vigan.05G144700.01	*Vigna radiata*	Cytoplasm	Unique to MCPI
Thioredoxin M-type chloroplastic	gi|734427533	*Glycine max*	Chloroplast	Unique to MCPI
Peroxisomal Catalase	Vigan.02G020900.01	Vigna radiata	Peroxisome	Unique to CPI
Ascorbate peroxidase	gi|1420938	*Vigna unguiculata*	Cytoplasm	Unique to CPI

Table 3 (continued)

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
Cell Structure				
Actin	gi|18532	*Glycine max*	Cytoplasm	0.440
Actin-3-like protein	gi|543176885	*Phaseolus vulgaris*	Cytoplasm	0.403
PREDICTED: golgin subfamily A member 5-like	gi|571466445	*Glycine max*	Golgi apparatus	Unique to MCPI
Stress and Defense Response				
Chaperonin 60 alpha subunit	gi|3790441	*Canavalia lineata*	Cytoplasm	0.425
Chaperonin-60 beta 4 isoform X4	gi|571434418	*Glycine max*	Chloroplast	0.328
Glycine-rich RNA-binding protein - 7 like	gi|356508388	*Glycine max*	Chloroplast	2.334
Glycine-rich RNA-binding protein	gi|5726567	*Glycine max*	Nucleus	2.411
PREDICTED: stromal 70 kDa heat shock-related protein	Vigan.04G318600.01	*Vigna radiata*	Cytoplasm	0.406

Table 3 (continued)

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
CPRD14 protein	gi|1854445	*Vigna unguiculata*	Golgi apparatus	Unique to MCPI
CPRD46 protein	gi|1853970	*Vigna unguiculata*	Cytoplasm	Unique to MCPI
Cysteine proteinase inhibitor	gi|288188	*Vigna unguiculata*	Cytoplasm/Secreted	Unique to MCPI
Trypsin inhibitor	gi|27529069	*Vigna unguiculata*	Cytoplasm/Secreted	Unique to MCPI
Cysteine proteinase RD21a	Vigan.03G287800.01	*Vigna mungo*	Lisosome	Unique to MCPI
Heat shock protein 90 kDa	gi|208964722	*Glycine max*	Cytoplasm	Unique to CPI
PREDICTED: heat shock cognate 70 kDa protein 2-like	Vigan.06G199300.01	*Vigna radiata*	Endoplasmic Reticulum/Mitochondrion	Unique to CPI
Heat shock protein 90-2	gi|351725976	*Glycine max*	Cytoplasm	Unique to CPI
PREDICTED: aldo-keto reductase family 4 member C9-like	gi|356496076	*Glycine max*	Chloroplast	Unique to CPI
Heat shock 70 kDa protein	Vigan.08G208500.01	*Vigna radiata*	Endoplasmic Reticulum/Mitochondrion	Unique to CPI
Energy and Metabolism				
Carbonic anhydrase	gi|422034738	*Vigna unguiculata*	Chloroplast	2.159

Table 3 (continued)

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
Phosphoglycerate kinase	gi|543177281	*Phaseolus vulgaris*	Cytoplasm	2.139
Vacuolar H+-ATPase subunit A partial	gi|66816974	*Vigna unguiculata*	Tonoplast	2.616
ATP synthase delta chain like protein	gi|543176740	*Phaseolus vulgaris*	Chloroplast	Unique to MCPI
Fructose-bisphosphate aldolase	gi|40457267	*Glycine max*	Cytoplasm	Unique to MCPI
Granule-bound starch synthase precursor	gi|145202752	*Vigna unguiculata*	Chloroplast	Unique to MCPI
Fructose-bisphosphate aldolase 1	gi|543176716	*Phaseolus vulgaris*	Chloroplast	Unique to MCPI
Glyceraldehyde-3-phosphate dehydrogenase C2	Vigan.02G218500.01	*Glycine max*	Mitochondrion	Unique to MCPI
Fructose-1,6-bisphosphate aldolase isozyme	gi|734430944	*Glycine max*	Cytoplasm	Unique to MCPI
NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	gi|357480133	*Medicago truncatula*	Cytoplasm	Unique to MCPI
Transketolase	gi|734414467	*Glycine max*	Chloroplast	Unique to CPI
Enolase	gi|351724891	*Glycine max*	Cytoplasm	Unique to CPI
PREDICTED: triosephosphate isomerase isoform X1	gi|571496004	*Glycine max*	Chloroplast	Unique to CPI

Table 3 (continued)

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
Isoflavone Biosynthesis				
Pterocarpan reductase	gi|555434199	*Phaseolus vulgaris*	Cytoplasm	Unique to CPI
PREDICTED: isoflavone reductase-like protein-like	gi|571458710	*Glycine max*	Cytoplasm	Unique to CPI
Nucleotide Binding				
ADP-ribosylation factor	gi|2689631	*Vigna unguiculata*	Endoplasmic Reticulum/Golgi apparatus	Unique to MCPI
PREDICTED: probable ADP-ribosylation factor GTPase-activating protein AGD11-like	gi|571489317	*Glycine max*	Endoplasmic Reticulum/Golgi apparatus	Unique to MCPI
Histone H2A homolog	gi|685035	*Phaseolus vulgaris*	Nucleus	Unique to CPI
PREDICTED: DEAD-box ATP-dependent RNA helicase 9-like	gi|571540128	*Glycine max*	Nucleus	Unique to CPI
Histone H2B	Vigan.06G198000.01	*Phaseolus vulgaris*	Nucleus	Unique to CPI
Putative histone H2A variant 3	gi|734330197	*Glycine max*	Nucleus	Unique to CPI

Table 3 (continued)

Protein Name	Accession (NCBInr)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
Photosynthesis and photorespiration				
PREDICTED: oxygen-evolving enhancer protein 3	Vigan.09G010500.01	*Phaseolus vulgaris*	Chloroplast	2.169
Chloroplast oxygen-evolving enhancer protein	gi|558695637	*Phaseolus vulgaris*	Chloroplast	2.195
Oxygen-evolving enhancer protein 1	gi|543176923	*Phaseolus vulgaris*	Chloroplast	3.148
Photosystem I subunit PsaD	gi|148372347	*Glycine max*	Chloroplast	2.829
Photosystem I-N subunit	gi|28629385	*Phaseolus vulgaris*	Chloroplast	2.199
Ribulose bisphosphate carboxylase	gi|21050	*Phaseolus vulgaris*	Chloroplast	3.471
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|349589843	*Vigna unguiculata*	Chloroplast	2.679
Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	gi|354460737	*Vigna unguiculata*	Chloroplast	2.972
Chlorophyll A/B binding protein 8	gi|543176847	*Phaseolus vulgaris*	Chloroplast	Unique to MCPI
Chlorophyll a/b-binding protein	gi|413968348	*Phaseolus vulgaris*	Chloroplast	Unique to MCPI

Table 3 (continued)

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
Phosphoglycolate phosphatase-like protein	gi|657378805	*Medicago truncatula*	Chloroplast	Unique to MCPI
PREDICTED: phosphoglycolate phosphatase 1B,	gi|356568529	*Glycine max*	Chloroplast	Unique to MCPI
Plastid-lipid-associated protein	Vigan.01G076400.01	*Vigna radiata*	Chloroplast	Unique to MCPI
Photosystem I reaction center subunit IV A	gi|357510347	*Medicago truncatula*	Chloroplast	Unique to MCPI
Photosystem II stability/assembly factor HCF136	gi|734402917	*Glycine max*	Chloroplast	Unique to MCPI
NADH-plastoquinone oxidoreductase subunit 2	gi|349589911	*Vigna unguiculata*	Chloroplast	Unique to MCPI
Photosystem I reaction center subunit III	gi|543177275	*Phaseolus vulgaris*	Chloroplast	Unique to MCPI
Photosystem II CP43 chlorophyll apoprotein	gi|349589903	*Vigna unguiculata*	Chloroplast	Unique to MCPI
Photosystem II D1 protein	gi|984735	*Vigna unguiculata*	Chloroplast	Unique to MCPI
Photosystem II protein D2	gi|349589854	*Vigna unguiculata*	Chloroplast	Unique to MCPI

Triosephosphate isomerase	gi|48773765	*Glycine max*	Chloroplast	Unique to MCPI
Photosystem I subunit III precursor	gi|8954295	*Vigna radiata*	Chloroplast	Unique to MCPI
PREDICTED: sedoheptulose-1,7-bisphosphatase like isoform 1	gi|356568652	*Glycine max*	Chloroplast	Unique to MCPI

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
Protein Metabolism				
PREDICTED: ruBisCO large subunit-binding protein subunit alpha	Vigan.07G208900.01	*Glycine max*	Chloroplast	Unique to MCPI
Ribosomal protein S5	gi|543176889	*Phaseolus vulgaris*	Chloroplast	Unique to MCPI
Ribosomal protein S8	gi|349589883	*Vigna unguiculata*	Chloroplast	Unique to CPI
Ribosome recycling factor	gi|540360868	*Arachis hypogaea*	Chloroplast	Unique to CPI
PREDICTED: 40S ribosomal protein S20-1-like	gi|356504833	*Glycine max*	Mitochondrion	Unique to CPI

Regulation Factor and RNA Processing				
EF-Tu	gi|18776	*Glycine max*	Cytoplasm/Nucleus	0.224
Elongation factor 1 beta	gi|308191651	*Vigna unguiculata*	Cytoplasm/Nucleus	0.303
Translation elongation factor Tu partial	Vigan.02G234700.01	*Phaseolus vulgaris*	Chloroplast	Unique to MCPI
PREDICTED: chloroplast stem-loop binding protein of 41 kDa	gi|356535978	*Glycine max*	Chloroplast	Unique to MCPI

Table 3 (continued)

Protein Name	Accession (NCBInr and VigGS)	Organism Reference	Cellular Compartment	Fold Change MCPI vs. CPI
eEF-1a2	gi|18765	*Glycine max*	Cytoplasm/Nucleus	Unique to CPI
Elongation factor 1-alpha	gi|543177265	*Phaseolus vulgaris*	Cytoplasm/Nucleus	Unique to CPI
Elongation factor Tu	gi|734407553	*Glycine max*	Chloroplast	Unique to CPI
Transferase				
PREDICTED: probable methyltransferase PMT3 isoform X3	gi|571445931	*Glycine max*	Chloroplast	Unique to MCPI

PREDICTED: probable methyltransferase PMT3-like isoform X1	gi|356536844	*Glycine max*	Chloroplast	Unique to MCPI
Putative methyltransferase PMT8	gi|734345453	*Glycine max*	Chloroplast	Unique to MCPI
Unknown				
Phosphoribosylglycinamide formyltransferase gi|12644307	gi|12644307	*Vigna unguiculata*	Chloroplast	Unique to MCPI

3.3 Protein-protein interaction networks in the cowpea plants exposed to CPSMV inoculation

It is well known that proteins display important roles in plant defense against pathogens. Usually, these proteins do not act isolated from each other, but function within complex interaction networks to form functional connections that are important for several defense mechanisms [35]. In the protein-protein network (String software) of differentially represented proteins of CPI inoculated with CPSMV, eEF-1A (A1) interacts with Actin (ACT 1), Actin-3-like protein (ACT 7), eEF-1B (AT5G12110), Histone H2A (HTA9), Histone H2B (HTB11) Putative histone H2A variant 3 (GAMMA-H2AX), and 40S ribosomal protein S20 (AT5G62300.1), all involved with protein synthesis and vesicle transport in cell. A link between this group of proteins with the Heat Shock Protein (HSP81-3), heat shock 70kDa protein (HSC70-1), heat shock protein 70B (HSP 70b), and heat shock protein 70-2 (cpHSP70-2) was also suggested. Furthermore, associations of proteins involved in the fermentative metabolism, as transketolase (AT2G45290), triosephosphate isomerase (TPI), and enolase (LOS2), were suggested (**Fig. 7 and Table 4**). For MCPI plantlets interactions of proteins related to photosynthesis and photorespiration, cell homeostasis redox, amino acid metabolism, and other pathways were predicted.

Likewise interactions of several proteins involved in both light and carbon assimilation reactions in photosynthesis such as oxygen-evolving enhancer proteins, chlorophyll A/B binding protein 1 (CAB1), NADH-Ubiquinone/plastoquinone (complex I) protein (NDHB), carbonic anhydrase 1 (CA1), ribulose-bisphosphate carboxylases (RBCL), and sedoheptulose-bisphosphatase (SBPAS) were depicted (**Fig. 8 and Table 5**). Moreover, in MCPI also occurred interactions between GOX and two CuZn-SOD (1 and 3), which are proteins involved in H_2O_2 generation, and between proteins involved in H_2O_2-scavenger, as peroxiredoxin-2E (AT3G52960), 2-cysteine peroxiredoxin B (AT5G06290), catalase 2 (CAT2) (AT4G35090), and Thioredoxin M-2 (ATHM2). GOX also interacted with serine hydroxymethyltransferase (SHM1), serine hydroxymethyltransferase 2 (SHM2), glutamine synthetase (GS2), and 2-phosphoglycolate phosphatase 1 (PGL1), all proteins related with the photorespiration pathway (**Fig. 8 and Table 5**).

Figure 7. Protein–protein interaction network analyzed by String software version 10.0. Network analyzed from differentially changed proteins in CPI cowpea leaves at 7 dpi with CPSMV. Different line colors represent types of evidence for each association: green line, neighborhood evidence; red line, 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.

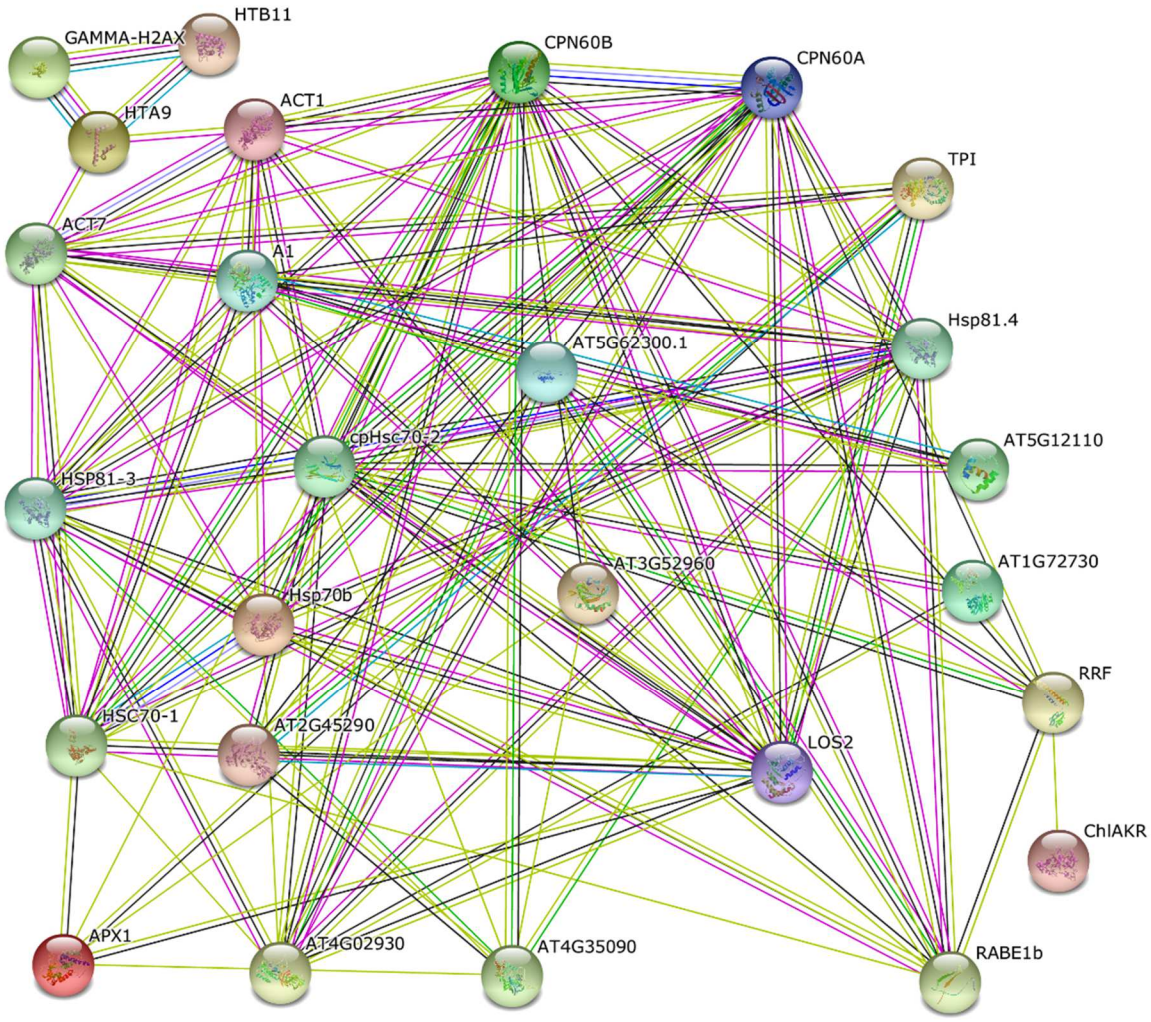


Table 4.

Abbreviations of the specific protein names in the protein-protein interaction network of differentially accumulated proteins in CPI (**Fig. 7**).

Abbreviation	Protein
Cell Structure	
ACT1	Actin 1
ACT7	Actin 7
Cell redox homeostasis	
APX1	L-ascorbate peroxidase
AT4G35090	Catalase 4
Nucleotide Binding	
HTA9	Histone H2A protein 9
GAMMA-H2AX	Gamma histone variant H2AX
HTB11	Histone H2B
Energy and metabolism	
AT2G45290	Transketolase
TPI	Triosephosphate isomerase
ChlAKR	Chloroplastic aldo-keto reductase
RNA factor and RNA processing	
AT4G02930	Elongation factor Tu
RABE1b	Elongation factor Tu
AT5G12110	Elongation factor 1-beta
AT1G72730	Translation initiation factor 4A
A1	Elongation factor 1-alpha
Stress and Defense response	
Hsp70b	Heat shock protein 70B
CPN60B	Chaperonin 60 beta
CPN60A	Chaperonin-60alpha
HSC70-1	Heat shock 70kda protein 1
cpHsc70-2	Heat shock protein 70-2
Hsp81.4	Heat shock protein 81.4

HSP81-3	Heat shock protein 81-3
LOS2	Low expression of osmotically responsive
Signaling and Carbohydrate Binding	
AT5G10530	Concanavalin A-like lectin kinase-like protein
AT5G65600	Concanavalin A-like lectin kinase-like protein
Proteins Metabolism	
AT5G62300.1	40S ribosomal protein S20-1
RRF	Ribosome-recycling factor
Isoflavone Biosynthesis	
AT1G75280	Isoflavone reductase-P3
AT1G75290	Isoflavone reductase-like protein

Figure 8. Protein–protein interaction network analyzed by String software. Network analyzed of differentially changed proteins in MCPI. Different line colors represent types of evidence for each association: green line, neighborhood evidence; red line, 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.

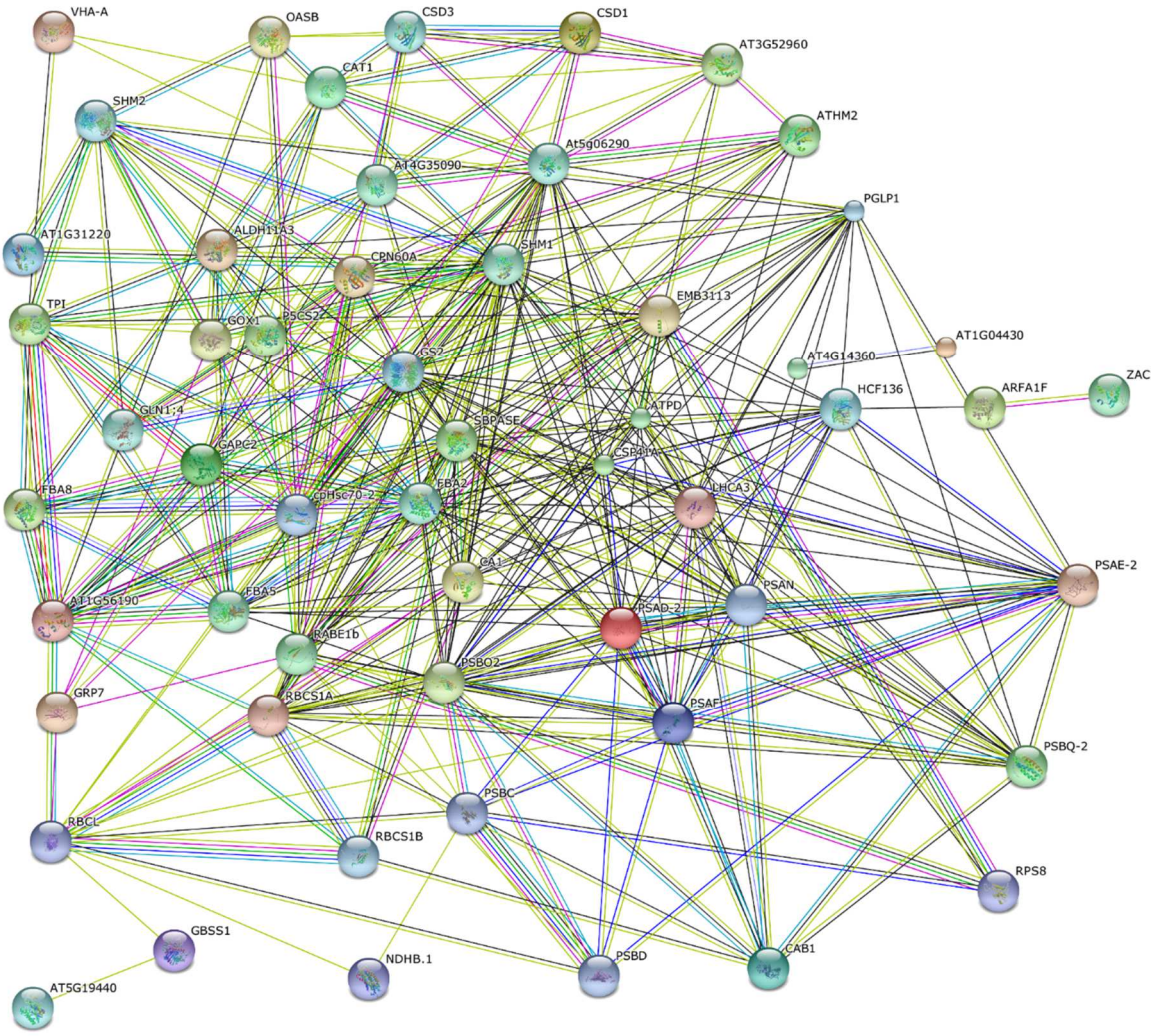


Table 5.

Abbreviations of the specific protein names in the protein-protein interaction network of differentially accumulated proteins in MPCI (**Fig. 8**)

Abbreviation	Protein
Photosynthesis and photorespiration	
PSAD-2	Photosystem I subunit D-2
CAB1	Chlorophyll A/B binding protein 1
PSAF	Photosystem I subunit F
LHCA3	Photosystem I light harvesting complex
RBCS1A	Ribulose bisphosphate carboxylase small chain
PSAE-2	Photosystem I subunit E-2
PSBO2	Photosystem II subunit O-2
CSP41A	Chloroplast stem-loop binding protein-41
SBPASE	Sedoheptulose-bisphosphatase
FIB	Fibrillin
PSBQ-2	Photosystem II subunit Q-2
AT5G19440	NAD(P)-binding domain-containing protein (326 aa)
HCF136	High chlorophyll fluorescence 136
RBCS1B	Rubisco small subunit 1B
PSAN	Photosystem I reaction center subunit N
PSBD	Photosystem II reaction center protein D
PSBC	Photosystem II reaction center protein C
RBCL	Ribulose-bisphosphate carboxylases
PGLP1	2-phosphoglycolate phosphatase 1 (362 aa)
NDHB.1	NADH-Ubiquinone/plastoquinone (complex I) protein
Cell redox homeostasis	
CSD1	Superoxide dismutase [Cu-Zn]
CAT1	Catalase 2
ATHM2	Thioredoxin M2
At5g06290	2-cysteine peroxiredoxin B
AT3G52960	Peroxiredoxin-2E

CSD3	Copper/zinc superoxide dismutase 3
GOX1	Glycolate oxidase 1
Nucleotide Binding	
ZAC	ADP-ribosylation factor GTPase-activating protein
ARFA1F	ADP-ribosylation factor A1F
Amino acid metabolism	
SHM1	Serine hydroxymethyltransferase
GLN1;4	Glutamine synthetase 1
SHM2	Serine hydroxymethyltransferase 2
GS2	Glutamine synthetase
OASB	Cysteine synthase
P5CS2	Gamma-glutamyl phosphate reductase

Energy and metabolism

FBA2	Fructose-bisphosphate aldolase 2
FBA5	Fructose-bisphosphate aldolase 5
TPI	Triosephosphate isomerase
FBA8	Fructose-bisphosphate aldolase 8
ATPD	ATP synthase delta-subunit
GAPC2	Glyceraldehyde 3-phosphate dehydrogenase
GBSS1	Granule-bound starch synthase
AT1G56190	Phosphoglycerate kinase
VHA-A	Vacuolar ATP synthase subunit A
CA1	Carbonic anhydrase 1
Stress and Defense response	
RD21A	Cysteine proteinase RD21a
GRP7	Glycine-rich RNA-binding protein 7
CYSB	Cystatin B
Transferase	
AT3G23300	Putative methyltransferase PMT1
AT4G14360	Putative methyltransferase PMT3
AT1G04430	Putative methyltransferase PMT8

4. Discussion

4.1 CPSMV interaction with cowpea

Plants are constantly challenged with diverse pathogens, but only a small number of them successfully invade and develop disease symptoms in a particular plant [36]. In this current study, the MCPI plantlets inoculated with CPSMV presented no visible symptoms of the viral disease at 7 DPI whereas severe typical symptoms were present in CPI plants (**Fig. 1A, B**). Indeed the presence of the viral particles detected by RT-PCR (**Fig. 1D, lane 2**) reveals the infection of CPI by CPSMV, which coincides with the presence of the typical severe disease symptoms (**Fig. 1B**). Contrary, the CPSMV particles were absent in the MCPI plantlets (**Fig. 1D – Lane 1**), which agrees with the lack of disease symptoms (**Fig. 1A**), and confirms that CPSMV failed to replicate in the mutagenized plantlets even after 3 months of planting at the flowering stage (**Fig. 1C**). Similarly, DiCarli et al. [37] did not detect disease symptoms or accumulation of CMV (*Cucumber Mosaic Virus,* Genus *Cucumovirus*) in a resistant transgenic tomato (*Solanum lycopersycum* cv. Micro-Tom) challenged with this virus, but the presence of severe symptoms and accumulation of CMV in a susceptible cultivar. Noteworthy, the seeds produced by the EMS mutagenized cowpea plants resistant to CPSMV generated plants there were susceptible to the virus, indicating that mutation was reversible.

One of the possibilities to explain MCPI resistance to CPSMV is that the EMS-induced cowpea mutagenizeds halt the virus invasion because the plantlets might have gained the capacity to produce Pattern Recognition Receptors (PRRs) that have the ability to perceive the pathogen or microbe-associated molecular patterns (PAMPs/MAMPs) and commence a basal defense known as PTI (PAMP-Triggered Immunity), referred as host resistance because it is often cultivar or accession specific. Other alternative would be through the R-gene mediated defense. EMS-induced cowpea mutagenizeds could be able to express disease resistance (R) genes that encode nucleotide-binding domain site and leucine-rich repeat (NBS-LRR)-type immune receptors, which recognize, directly or indirectly, effector proteins synthesized by pathogens to suppress PTI, and initiate the Effector Triggered Immunity (ETI) [38-41] that involve participation of several other proteins. Recently Kørner et al. [42]

suggested that PTI is important for Arabidopsis resistance to viruses because they found evidence that viral MAMPs are directly or indirectly recognized by PRR which initiates a signaling cascade that culminates with PTI [42]. A third possibility, is that the mechanism of plant resistance results from the lack of interactions between plant and viral factors required for triggering translation of viral proteins. The replication of viral nucleic acid due to the absence or mutation of the proper host factor that leads to an incompatible relationship in which no or very limited virus replication occurs in the plant that shows no symptoms or extremely limited necrosis [43]. For example, various studies have shown that effective recessive resistance of various plants to Potyvirus resides in the inability of VPg (Viral Genome-linked Protein) to interact with the eukaryotic translation initiation factor eIF4E or eIF(iso)4E of the plant due to mutations in the genes that code for these proteins [44,45]. This incapacity of interaction occurs as the result of any replacement of single or multiple amino acid residues exposed on the surface of the eukaryotic translation initiation factor proteins regardless whether in the domain of interaction with 5' 7-methylguanosine cap (7-methyl-GTP cap) or not [46]. In most cases, the result is the inhibition of multiplication of viral material. Some studies showed that replacement of only one amino acid residue in eIF4E is sufficient to confer resistance, as in the case of replacing alanine with proline residue in lettuce, or valine with glutamine residue in pepper [47,48]. In other cases, the mechanism of the resistance breaking resulted from the change in more than one amino acid residues, as in common bean (*Phaseolus vulgaris*) against the Potyvirus BCMV (*Bean Common Mosaic Virus* genus *Potyvirus*) [49]. Although, the majority of cases of recessive resistance is conferred by mutations of genes coding for the translation initiation factors eIF4E and eIF(iso)4E [17,45,50], as well as in both affecting their interactions with VPg, mutations in other components of the family eIF4F [eIF4G and eIF(iso)4G], also affect the replication in some species of virus [51-54].

4.2 Changes in Proteomic profiles of cowpea leaves in response to CPSMV

Comparative proteomic analysis of CPI and MCPI plantlets at 7 DAI with CPSMV showed 99 proteins significantly altered, amongst which 52 proteins were detected exclusively in MCPI, and 24 proteins in CPI (**Fig. 3 and Table 3**). Alterations

in the proteome of virus-infected compared with uninfected control plants have been previously reported by other researchers [55,56].

The majority of the identified proteins in MCPI and/or CPI matched those of *Glycine max* (39) and/or *Vigna unguiculata* (23). The remaining protein matched those of *Phaseolus vulgaris* (21), *Vigna radiata* (10), *Medicago truncatula* (3), *Arachis hypogaea* (1), *Vigna mungo* (1), and/or *Canavalia lineata* (1) (**Table 3**), all plant species that belong to the *Fabaceae* family in which cowpea (*V. unguiculata*) is included. NCBI's non-redundant *Viridiplantae* database search and VIgGs [24] were used to identify proteins matched to those of *V. unguiculata*, which showed low number of annotated proteins when compared with *G. max* or *P. vulgaris*. Nevertheless, all differentially represented proteins in CPI and MCPI are involved in various biochemical and/or physiological processes (**Fig. 3**), which denote the complexity of the genetic reprogramming that susceptible and resistant cowpea counterparts can activate/repress when challenged with CPSMV.

4.3 Biological classification of the differentially represented cowpea proteins

4.3.1 Amino acid and protein metabolism

Six and five differentially represented cowpea proteins are involved in amino acid and protein metabolism, respectively (**Table 3**). Interestingly, all six proteins involved in the amino acid metabolism were represented only in MCPI plantlets as, for example, two glutamine synthetase isoforms (one of chloroplast and other mitochondrial), which increased in abundance compared to CPI (**Table 3**). Ward et al. [57] reported that inoculation of tomato plants with *Pseudomona syringae* pv. tomato induced high accumulation of several amino acid types including glutamine in comparison with control.

Another protein, Delta 1-pyrroline-5-carboxylate synthetase, an enzyme involved in the proline biosynthesis, also was over-represented in MCPI plantlets. Proline is an important amino acid for resistance to biotic and abiotic stresses in plants. In *Arabidopsis,* proline accumulation was associated to HR after pathogen inoculation [58]. Moreover, transcriptomic analysis in *Arabidopsis* revealed up-regulation of

several genes involved with the amino acid metabolism in response to pathogens, suggesting important roles of amino acids in plant defense responses [57-58]. A recent proteomics study on rice (*Oryza sativa*) RSV (*Rice Stripe Virus,* Genus *Tenuivirus*) interaction showed perturbation in the amino acid metabolism in susceptible rice plants [55].

The MCPI plantlets also showed increased abundance of two mitochondrial isoforms of Serine hydroxymethyltransferases (SHMT), in comparison with CPI. SHMT is an enzyme that catalyzes the conversion of glycine to serine in photorespiration [59]. Recently, our research group showed increased level of SHMT during the incompatible interaction between a resistant cashew plant (clone BR 226) and the fungus *Lasiodiplodia theobromae* suggesting a role of this protein in defense [60]. These over-representations of some proteins in MCPI involved with the amino acid metabolism suggest that the nitrogen remobilization is important for plants to cope with the CPSMV attack.

In relation to the 5 proteins identified as involved in the protein metabolism, 2 were exclusively represented in MCPI, and 3 in CPI plantlets. The 3 proteins were exclusively represented in CPI plantlets were ribosomal protein S8, ribosome-recycling factor (RRF), and 40S ribosomal protein S20 like protein (Table 3). The 40S ribosomal proteins S8 and S20 are part of the 40S ribosomal subunit responsible for binding to the eukaryotic translation initiation factors eIF1 and eIF1A, and recruitment of the plant Cap-mRNA to start mRNA translation [61-63]. As previously mentioned CPMSV has a VPg at the 5'end of both the RNA1 and RNA2 of the virus genome. VPg proteins are capable to interact with the 40S ribosomal protein subunits of plants recruiting the initiation factors of the translation complex to viral RNA, resulting in depletion of these factors for host mRNA translation and favoring viral mRNA translation and thus disease development [64,65].

The ribosome-recycling factor (RRF) is an important protein that disassembles the post-termination complex at the last step of translation releasing the ribosomes from mRNAs by splitting them into their subunits at the termination codon (UAA, UAG, or UGA) leaving the subunits free to bind new mRNA (ribosome recycling) that is translate into a new protein molecule [66]. Perhaps, RRF was induced to increase in abundance in the CPI group because the virus recruit the plant ribosomes to translate

the viral mRNAs, and this recruitment acts as a feedback signal that leads the plant to synthesize spare amounts of RRF to assembly further ribosome subunits as an attempt to restore translation of the plant mRNAs into its own proteins. It is well known that the protein metabolism in plants during viral infection is affected because viruses redirect the translation machinery of their host to favor viral protein synthesis leading to a kind of "*shutoff*" of plant protein synthesis, [9,63], favoring virus proliferation and disease establishment in susceptible plants.

4.3.2 Cell structure, carbohydrate binding, and signaling

Actin and actin-3-like proteins, involved in cell structure, were identified in MCPI and CPI plantlets (**Table 3 and Table S1**)**.** However, in MCPI they were both down-represented in comparison to CPI. Recent works strongly suggest that actin is a plant host factor that interact with viral movement proteins favoring viral intracellular movement by increase the size exclusion limits of plasmodesmata thus improving viral movement and infection [67,68]. For example, *N. benthamiana* leaves treated with LatB, a disruptor of actin filaments, reduced the cell-to-cell movement and replication efficiency of three distinctly related viruses: TMV (*Tobacco Mosaic Virus*, Genus *Tobamovirus*), PVX (*Potato Virus X*, Genus *Potexvirus*) and TBSV (*Tomato Bushy Stunt Virus*, Genus *Tombusvirus*) [69]. Therefore, it is suggested that in CPI plantlets, CPSMV induces up-accumulation of actin proteins to improve its movement and replication efficiency during the infection process, whereas the lower levels in the resistant MCPI group help the plantlets to impair viral establishment.

Concerning to carbohydrate binding and signaling, two proteins deserve special attention: Calcinerium B-like interacting protein kinase (CIPK); and L-type lectin-domain containing receptor kinase (LecRK), both exclusively detected in MCPI but not in CPI plantlets at 7 DPI (**Table 3**). CIPK are a group of serine/threonine protein kinases that interact with the plant Ca^{2+} sensors, calcinerium B–like (CBLs) proteins, which plays a key role in decoding calcium signaling in response to both abiotic and biotic stresses [70]. Recently, two studies described new functions to CBL-CIPK in plant defense against virus. First, CBL-like proteins interact with viral double-stranded RNA (dsRNA) inducing degradation of viral dsRNA [71]. For instance, viruses can produce significant amounts of dsRNA with a genome consisting of positive-strand

RNA, dsRNA, or DNA [72]. Second, CIPK7 from *A. thaliana* affected TuYV (*Turnip Yellow Virus,* Genus *Tymovirus*) long-distance movement and accumulation in systemic leaves by interacting with the viral movement protein preventing both the systemic disease establishment and virus exit from infected cells [73].

Regarding to LecRK, this protein plays crucial roles in plant innate immunity and adaptive response to various stresses. Perception and transduction of environmental stimuli are largely governed by receptor-like kinases [74,75]. Overexpression of LecRK-IX.1 or LecRK-IX.2 in *A. thaliana* and *N. benthamiana*, respectively, increased resistance against pathogens and could be involved in cell death [75].

4.3.3 Nucleotide binding proteins and regulation factors/RNA processing proteins

Six proteins classified into the family of nucleotide binding proteins were identified (**Table 3**). However, to our viewpoint only one requires attention - PREDICTED: DEAD-box ATP-dependent RNA helicase 9-like (DDX) which was exclusively detected in CPI plantlets (**Table 3**), but was absent in MCPI. DDX plays important roles in RNA metabolism; however, it has also been recognized as a host factor that enhances virus replication in plants. Huang et al. [76] showed that *Arabidopsis* DDX interacts with the potyviral VPg protein favoring PPV (*Plum Pox Virus*, Genus *Potyvirus*) and TuMV (*Turnip Mosaic Virus*, Genus *Potyvirus*) replication. These authors [76] using the atrh8 *Arabidopsis* mutagenized, that did not express DDX, demonstrated that these plants in addition to have normal development did not allow PPV nor TuMV replication, strongly suggesting that the gene atrh8 that codes DDX is not necessary for plant development, but is required for viral replication.

Viruses can infect and complete they life cycle in a susceptible host and easily spread systemically to developed systemic disease. Compatible plant-virus interaction involves effective use of necessary host factors and suppression of the host defense mechanisms [57,77]. Seven proteins related to Regulation factors/RNA processing proteins were identified in both cowpea studied plantlets at 7 DPI with CPSMV. EF-Tu and elongation factor 1 beta (eEF1B) decreased in abundance ($p<0.05$) in the MCPI

plantlets in comparison with CPI (**Table S1**). In addition, two isoforms of eEF-1A1 and the Elongation factor 1-alpha (eEF1A) were present only in the CPI plantlets (**Table 3**). Several studies report that eEF1A and eEF1B proteins are host factors largely used by virus to enhance the replication process and cell-to-cell movement [9,63,77-80]. Hwang et al. [78] reported that the nucleotide exchange factor eEF1Bβ one of the two components of the eukaryotic translation elongation factor 1 (eEF1) plays essential roles in the multiplication of PVX in *N. benthamiana* by physically interacting with the Triple Gene Block 1 (TGBp1) protein encoded by this virus. Actually, these authors showed that in the *eEF1Bβ*-, *eEF1Bɣ*-, and *eEF1A*-silenced plants accumulation of a green fluorescent protein (GFP)-tagged PVX significantly decreases, suggesting that *eEF1Bβ* could be a potential target for engineering virus-resistant plants [78].

It has long been known that eEF1A also interacts with the 40S small ribosomal subunit and with actin [77,80]. Interestingly, 40S small ribosomal subunit protein and actin showed increased levels in CPI plantlets (**Table 3**) in comparison to MCPI. In addition, the protein-protein interaction network shows that it is possible interaction between these proteins with eEF1A. Such interactions (40S ribosome subunit + Actin + eEF1A + eEF1B) in CPI plantlets, probably, favor the replication process, protein synthesis, cell-to-cell movement and systemic movement of CPSMV and thus the virus disease establishment (**Fig. 7**).

4.3.4 Energy and metabolism

Over-representation of proteins within these classes predominated (10) in MCPI plantlets in relation to CPI (**Table 3**) suggesting the importance of energy (ATP and NADPH) production to trigger defense responses towards viral infection. In plants, activation of defense responses has a high cost in mobilizing metabolic changes to overcome or suppress the pathogen attack [81]. In this current work, changes in the modulation of proteins related to glycolysis and gluconeogenesis metabolic pathways were noticed. Glycolysis and gluconeogenesis are antagonistic pathways of carbohydrate metabolism that occur under fine coordinated regulation. Interestingly, glyceraldehyde 3-phosphate dehydrogenase C2 subunit (GADPH) and phosphoglycerate kinase (PGK) that are both involved in glycolysis and gluconeogenesis, and three fructose-1,6-bisphosphatase aldolase (1,6-ALD) isoforms

that participate in gluconeogenesis were up-represented in MCPI plants (**Table 3**) at 7 DPI with CPSMV. Maintenance of high cellular respiration rates to provide energy is imperative to trigger and sustain resistance responses. Therefore, increased GAPDH levels upon CPSMV infection may enhance plant respiration rates to provide the additional energy needed to respond to viral infection [82]. Noteworthy, several evidences support the nonglycolytic functions of GADPH, including RNA synthesis, and PCD and immunity response to various pathogens including viruses [83]. Prasanth et al. [84] showed that GAPDH interacts preferentially with positive strand RNA and inhibits viral replication. This is an interesting fact because CPSMV is a positive strand RNA virus, and it is plausible to speculate that GADPH could also have the ability to interact and inhibit RNA replication preventing CPSMV infection, in addition to participate in the carbohydrate metabolism to provide energy for MCPI plantlets. Havelda et al. [85] showed that *N. benthamiana* when infected with PVX (*Potato Virus X,* Genus *Potexvirus*), CymRSV (*Cymbidium Ring Spot Virus,* Genus *Potexvirus*), and TMV (*Tobacco Mosaic Virus,* Genus *Tobamovirus*), three viruses belonging to different genus had a decreased GADPH level, which favored virus replications and enhanced disease symptom severity.

4.3.5 Stress and defense responses

Several defense proteins increased in abundance in MCPI plantlets in comparison to CPI, but four of them deserve particular attention: two Glycine-rich RNA-binding protein (GRP7) isoforms, a cysteine proteinase inhibitor, and a trypsin inhibitor. Plant protease inhibitors are classified in the family 6 (PR-6) of PR-proteins (pathogen-related proteins) [86]. During viral infection, translation of the RNA-1 of CPSMV result in a polyprotein precursor, which is cleaved by a viral protease to produce viral proteins [87]. In connection with this viral polyprotein processing, it is plausible to suggest that the enhanced level of PR-6 might play an important role in the resistance of MCPI plantlets against CPSMV, probably by inhibiting the proteolytic cleavage of the virus polyprotein and preventing CPSMV replication. The role of protease inhibitor in plant defense against the potyviruses TEV (*Tobacco Etch Virus*) and PVY (*Potato Virus Y*) was previously reported [88] in transgenic tobacco plants engineered to produce oryzacystatin I. The authors explain that the transgenic tobacco plants that shown overaccumulation of oryzacystatin I inhibits viral replication,

because viruses requires a cysteine proteinase activity in their replication mechanisms. Thereby, there was a clear direct correlation between the oryzacystatin I levels, papain inhibition class, and resistance to both viruses in all tobacco lines tested.

Concerning to RNA binding proteins, MCPI plantlets up-accumulated two GRP7 isoforms. Plants often use RNA binding proteins for defense against viral infections. After invading the plant cell, exposed viral RNAs are susceptible to a variety of mechanisms to inhibit viral RNA replication by plant RNA binding proteins, like GRP7 [89,90]. For example, over-accumulation of AtGRP7 conferred resistance to *A. thaliana* against TMV (*Tobacco Mosaic Virus,* Genus *Tobamovirus*) [89]. Another important function attributed to GRPs is interaction with proteins involved with lignin and callose biosynthesis and deposition, resulting in cell wall strengthening and reduction in size-exclusion limits of plasmodesmata, towards avoiding virus spreading throughout the plant tissues [91,92].

Concerning to stress response proteins, most of them were identified as HSP70 and HSP90, but exclusively detected in CPI plantlets (**Table 3**). Several studies have demonstrated that HSPs (HSP70 and HSP90 families) are plant host factors that favor virus replication in plants [93,94]. For instance, Mine et al. [94] found that HSP70 and HSP90 interact with the p27 protein from RCNMV (*Red Clover Necrotic Mosaic Virus,* Genus *Tombusvirus*) to form a virus-encoded component of the 480-kDa replicase complex supporting RNA replication. The authors suggest that HSP70 and HSP90 regulate different steps in the assembly of the RCNMV replicase complex that are necessary for virus replication [94]. Interestingly, in our present study both HSP70 and HSP90 increased in abundance only in CPI plantlets in relation to MCPI plantlets, in which these proteins were probably below the LC–MS/MS detection threshold. Therefore, it is suggested that both HSP70 and HSP90 also favor CPSMV replication and accumulation in CPI plantlets.

4.3.6 Cell redox homeostasis

Following pathogen recognition, via transmembrane PRRs that respond to MAMPS or PAMPs, or NB-LRR proteins encoded by R genes that works largely inside the cell [11], inducing ROS generation, particularly H_2O_2 that is a major signaling molecule, activates or represses several signaling pathways involved in various

biological processes including responses to biotic stresses, particularly, viruses [12,36,95]. On the other hand, to prevent damage caused by excessive ROS production, plants have antioxidant systems including the enzymes SOD, CAT, and APX, in addition to peroxiredoxins (Prxs), glutaredoxins (Grxs), and thioredoxins (Trxs), which exist in multiple isoforms [96].

In this current study, two CuZn-SOD and GOX that contribute to H_2O_2 generation increased in abundance in MCPI Plantlets. This is coincident with higher H_2O_2 accumulation in MCPI in comparison with CPI plantlets (**Fig. 4**). Kundu et al. [97] reported over-accumulation of SOD enzymes accompanied by increased H_2O_2 levels in *V. mungo* genotypes resistant to MYMIV (*Mungbean Yellow Mosaic India Virus,* Genus *Begomovirus*). In parallel with increased H_2O_2 levels, PCD was more prominent in MPCI than in CPI plantlets (**Fig. 5**), probably mediated by H_2O_2. PCD is an important plant defense mechanism against biotrophic pathogens, like viruses. Indeed, during infection, PCD blocks virus spreading to uninfected cells and tissues preventing the systemic disease establishment [10-12].

In concert with increased H_2O_2 levels, MCPI plantlets showed increased abundance of H_2O_2 scavenger enzymes, such as CAT-2 and 2-CysPrxA, both confirmed by qPCR (**Fig. 6**), Prx2E, and TrxM (Table 3) suggesting the importance of these proteins in regulating H_2O_2 levels and maintaining redox homeostasis avoiding damage to plant tissue. It is possible that enhanced 2-CysPrxA, Prx2E and TrxM contents (**Table 3**) in MCPI plants is involved in preventing the oxidative damage of the photosynthetic machinery caused by ROS produced in chloroplasts, as previously indicated [98,99], during the response to CPSMV infection. Increased abundance of Prxs and Trxs during the incompatible interaction between *V. mungo* and MYMIV improved redox homeostasis associated with high tolerance to the virus [100]. In addition, Baier and Dietz [99] reported that the photosynthetic machinery needs high levels of Prxs to protect it from oxidative damage. Recently it was verified by our research group that Prxs and Trxs increased in abundance in response to the incompatible interaction between cowpea (genotype BR-3) and *Colletotrichum gloeosporioides* [22], suggesting the role of these antioxidant proteins in cowpea defense against pathogens (fungi and viruses), probably, by improving excess H_2O_2 scavenge and protecting the photosynthetic machinery toward damage cause by ROS.

4.3.7 Proteins involved in photosynthesis and photorespiration

Photosynthetic reactions in photosystem II (PSII) generates ROS in chloroplasts. Proteomic studies have shown that photosynthesis-related proteins decrease in abundance in virus-infected plants [37,97]. Therefore it is consistent that biotrophic pathogens, like viruses, which require host cells to be alive, try to decrease photosynthesis and related process [81,101] to avoid PCD. It has been reported that viruses can reduce the photosynthetic capacity during a compatible interaction by accumulation of CP inside the chloroplasts, which affects the PSII core complex, inhibiting its function or interfering with the chlorophyll metabolism [102-105].

In this current work, all the identified photosynthesis and photorespiration related proteins increased in abundance in MCPI plants in comparison to CPI (**Table 3**). Oxygen-evolving enhancer proteins is essential for the normal function of chloroplast and, consequently, for the photosynthetic activity. Reduction of oxygen-evolving enhancer protein levels in CPI induced by CPSMV infection must be associated with the reduction of photosynthetic activity, chlorophyll production, and chlorosis with consequently reduction in chlorophyll index (**Table 6 and 7**) [55,106]. Recently, it was reported that oxygen-evolving enhancer proteins might have direct action against the virus by inhibiting viral replication. For example, Balasubramaniam et al. [107] showed that the PSII oxygen-evolving enhancer proteins specifically interacts with the coat protein (CP) of AMV (*Alfalfa Mosaic Virus,* Genus *Begomovirus*), inhibiting replication, which denotes a direct role in antiviral defense.

Another important protein for the functional integrity of chlorophyll and chloroplast is the chlorophyll a/b-binding protein. Wang et al. [55] reported that infection of rice with RSV induced a decrease in abundance of chlorophyll a/b-binding proteins leading to disruption of chlorophyll biosynthesis. In our present work, two isoforms of chlorophyll a/b-binding protein in MCPI plants were identified, although absent in CPI plantlets (**Table 3**). It has been reported that virus infection of susceptible plants causes reduction in chlorophyll interacting proteins and consequent reduction of the chlorophyll content, which leads to decreased photosynthesis index accompanied by mosaic formation [104,105]. All together decrease in abundance of oxygen-evolving enhancer proteins and chlorophyll a/b-binding proteins in CPI

induced by CPSMV infection might be related to the reduction of chlorophyll content and thus the appearance of the typical mosaic symptoms. To reinforce this suggestion, quantification of chlorophyll content in both experimental groups showed that CPI plants have significant ($p \leq 0.05$) reduction (2.53-, 1.77-, and 2.11-fold) in total chlorophyll, chlorophyll *a*, and chlorophyll *b* content, respectively, in comparison with MCPI plantlets (**Table 6**). These findings corroborate the manifestation of chlorosis and mosaic symptoms in CPI (**Fig. 1B**) likely caused by decreased levels of Oxygen-evolving enhancer proteins and chlorophyll a/b-binding proteins induced by CPSMV infection.

In addition, MCPI up-accumulated photosystem II D1 (PSIID1) and photosystem II D2 (PSIID2) proteins related to PSII and chloroplast integrity as these proteins were not identified in CPI group. Increase in abundance of PSIID1 and PSIID2 indicates a high turnover of these proteins in association with prevention of photoinhibition (**Table 7**) induced by CPSMV. On the other hand, the low levels of PSIID1 and PSIID2 in CPI, below the LC–MS/MS detection threshold, led to photoinhibition of PSII and consequently inhibition of photosynthesis. Indeed, it was noticed a significant decrease (1.94-fold) of PSII activity in CPI plantlets as compared with MCPI (**Table 7**). Thus, the very low levels of PSIID1 and PSIID2 associated with low PSII activity in CPI plantlets, strongly suggest that CPSMV induces photoinhibition of PSII, which might limits energy production by light reactions. Wang et al. [108] reported that tobacco (*N. tabacum*) tolerant to TMV infection increased PSIID1 accumulation, whereas in TMV-infected susceptible plants the PSIID1 content decreased, suggesting that PSIID1 is essential for TMV tolerance in tobacco.

Four proteins related to the Calvin-Benson cycle were up-represented in MCPI plantlets: Ribulose bisphosphate carboxylase; Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit; Ribusco small subunit; and PREDICTED: sedoheptulose-1,7-bisphosphatase like isoform 1 (**Table 3**). Increased abundance of these proteins suggests higher net CO_2 assimilation and stomatal conductance in MCPI when compared to CPI plantlets. Our data confirm these findings as both net CO_2 assimilation and stomatal conductance at 7 DPI increased 1.77- and 1.66-fold, respectively, in MCPI in comparison with CPI plantlets (**Table 7**). Increased CO_2 assimilation is likely involved with the higher demand for assimilates, such carbon skeletons, required by plants to establish defense responses [109].

Up-accumulation of photosynthetic proteins in MCPI plantlets challenged with CPSMV suggests that resistant plants evoke mechanisms to protect the photosynthetic machinery not only to preserve, but improve the photosynthetic performance required to provide energy (ATP) and reducing potential (NADPH) to support the primary metabolism and thus allowing plants to counterattack the virus infection. In addition, H_2O_2 over-production by photorespiration probably contributes to plant defense against the virus by inducing PCD to prevent viral spreading, and by inducing expression of defense related genes.

During infection, many virus induce perturbations in several cellular process important to plant defense, such energy production, redox homeostasis, photosynthesis, amino acid and protein metabolism provided by primary metabolic pathways, to avoid plant defenses mechanisms and support disease establishment. However, during host resistance, several cellular metabolic process involving subcellular organelles to form a complex interaction network to prevent infection [81].

Here, we showed the complex interaction of several cellular organelles and proteins related to a variety of molecular functions, involved in MCPI resistance to CPSMV (**Fig. 9**). The MCPI plants up-represented several proteins that are involved in plant defense to virus infection and down-represented host proteins used by virus to improve replication and disease establishment. Thus, a complex pattern of proteins changed in MCPI plants during CPSMV infection. The global cellular responses of mutagenized resistant cowpea plants to CPSMV infection are summarized in **Fig. 9** according to putative functions of the differentially represented proteins.

Table 6.
Effect of CPSMV infection on chlorophyll content of *V. unguiculata,* genotype CE-31, leaves obtained from 0.04% EMS treated and untreated cowpea seeds

Groups	Total Chlorophyll mg g^{-1} FW	Chlorophyll *a* mg g^{-1} FW	Chlorophyll *b* mg g^{-1} FW
MCPI plants	1.52 ± 0.016^{a}	0.80 ± 0.012^{a}	0.72 ± 0.002^{a}
CPI plants	0.60 ± 0.013^{b}	0.45 ± 0.006^{b}	0.34 ± 0.009^{b}

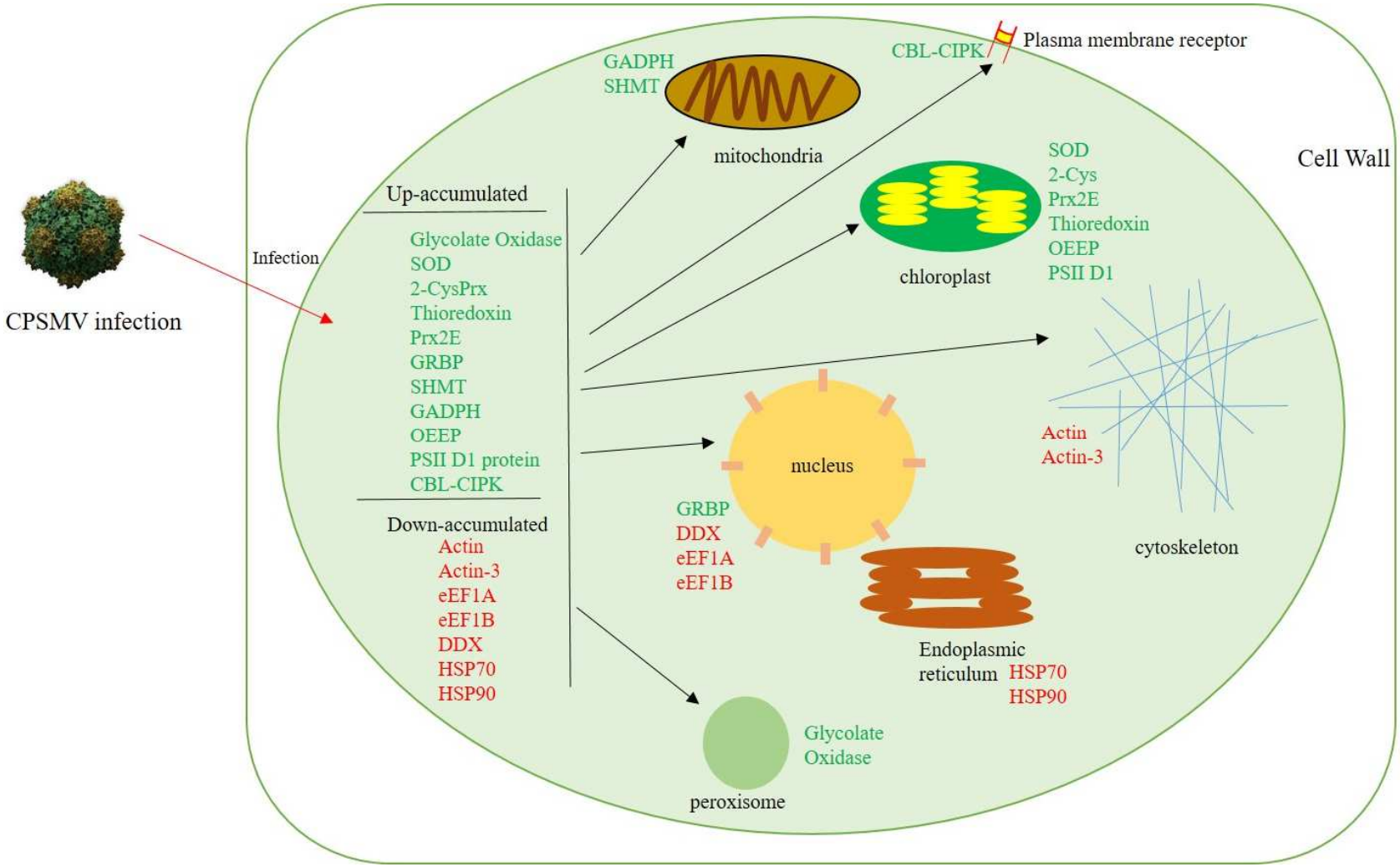
Values are mean ± standard deviation (SD); data followed by same alphabets are not significantly different at ($p \leq 0.05$) according to Tukey test.

Table 7.
Effect of CPSMV infection on physiological process of *V. unguiculata,* genotype CE-31, leaves obtained from 0.04% EMS treated and untreated cowpea seeds

Groups	Quantum Efficiencies of PSII (ΔF/FM')	Net CO_2 Assimilation (P_N)	Stomatal Condutance (gs)
MCPI plants	0.229 ± 0.007^{a}	10.55 ± 0.070^{a}	0.291 ± 0.028^{a}
CPI plants	0.118 ± 0.007^{b}	5.95 ± 0.330^{b}	0.175 ± 0.007^{b}

Values are mean ± standard deviation (SD); data followed by same alphabets are not significantly different at$p \leq 0.05$ according to Tukey test.

Figure 9. The global cellular responses of MCPI plants to CPSMV infection. Green and red names indicate proteins that are up- and down-represented respectively.



5. Conclusion

In summary, the treatment of cowpea (CE-31 genotype) seeds with EMS yielded mutagenized, CPSMV resistant plantlets (MCPI). This work used a label-free comparative proteomic approach (LC-ESI-MS/MS) as an attempt to established biochemical and physiological differences associated with MCPI resistance and CPI susceptibility to CPSMV. Changes in several metabolic pathways are potentially associated with MCPI resistance and CPI susceptibility to CPSMV. These changes arise from the findings that 31 key host proteins were down-represented in MCPI whereas 68 up-regulated in MCPI plantlets after CPSMV challenge. Obviously, these preliminary results are not conclusive; however, they provide clues for further studies towards elucidating the molecular and physiological mechanisms underlying the resistance and/or susceptibility traits of cowpea to CPSMV.

Conflicts of interest

The authors declare no conflict of interest.

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Author contributions

P. F. N. Souza, F. D. A. Silva, I. M. Vasconcelos, and J. T. A. Oliveira, conceived the study and wrote the manuscript (with input from all authors); P. F. N. Souza, F. D. A. Silva, I. M. Vasconcelos, and J. T. A. Oliveira performed all biochemical assays and interpreted the experimental data; K. S. Oliveira and O. L. Franco performed all proteomic analysis and interpreted the experimental data; J. A. G. Silveira, produced and interpreted all physiological data and participated. All authors read and approved the final version of the manuscript.

Supplementary Files

Supplementary Table S1.
Overlapping protein between MCPI x CPI

Protein Name	Peak Area						Fold Change MCPI/CPI			Mean Fold Change MCPI/CPI
	CPI			MCPI						
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	
Cell Redox Homeostasis										
Copper/zinc superoxide dismutase	22988.294	22429.732	22227.364	132758.899	132072.651	132255.043	5.775	5.888	5.950	5.871
2-Cys peroxiredoxin BAS1-like	7648.223	7465.144	7331.910	19710.648	19683.186	19354.540	2.577	2.637	2.640	2.618
Peroxiredoxin-2E	15684.833	15587.556	15629.285	42434.381	42669.003	42591.800	2.705	2.737	2.725	2.723
Cell Structure										
Actin	14529.628	14563.079	14719.294	6601.922	6358.291	6336.790	0.454	0.437	0.431	0.440
Actin-3-like protein	53089.433	54189.911	53157.626	21501.773	21854.854	21303.355	0.405	0.403	0.401	0.403
Stress and Defense Response										
Chaperonin 60 alpha subunit	31383.455	31520.100	31665.960	13283.898	13636.470	13254.667	0.423	0.433	0.419	0.425
Chaperonin-60 beta 4 isoform X4	23367.696	22698.254	22364.170	7449.442	7213.376	7771.954	0.319	0.318	0.348	0.328
Glycine-rich RNA-binding protein - 7 like	10561.669	10352.270	10525.248	24417.92922	24740.15486	24218.06247	2.312	2.390	2.301	2.334

Glycine-rich RNA-binding protein	25622.568	25437.851	25234.235	60990.64983	61313.22656	61625.03667	2.380	2.410	2.442	2.411
PREDICTED: stromal 70 kDa heat shock-related protein	30142.341	30526.348	30074.036	12367.073	12351.057	12151.318	0.410	0.405	0.404	0.406
Energy and Metabolism										
Carbonic anhydrase	14315.330	14291.633	14270.313	30994.654	30964.329	30634.498	2.165	2.167	2.147	2.159
Phosphoglycerate kinase	12112.161	12464.945	12372.441	26226.411	26541.958	26250.749	2.165	2.129	2.122	2.139
Vacuolar H+-ATPase subunit A partial	8252.806	8241.240	8290.558	21421.747	21456.017	21968.337	2.596	2.603	2.650	2.616
Photosynthesis and photorespiration										
PREDICTED: oxygen-evolving enhancer protein 3	15572.552	15363.407	15590.657	33411.533	33769.981	33747.775	2.146	2.198	2.165	2.169
chloroplast oxygen-evolving enhancer protein	10129.803	10276.803	10154.563	22501.686	22321.000	22269.355	2.221	2.172	2.193	2.195
oxygen-evolving enhancer protein 1	14222.803	14996.336	14756.222	46088.756	46202.481	46092.403	3.240	3.081	3.124	3.148

photosystem I subunit PsaD	6643.558	6527.214	6306.658	18163.658	18649.964	18266.083	2.734	2.857	2.896	2.829
photosystem I-N subunit	55693.801	55398.223	55921.030	122223.875	122617.547	122462.120	2.195	2.213	2.190	2.199
Ribulose bisphosphate carboxylase	22809.265	22110.293	22138.368	77625.392	77474.653	77644.587	3.403	3.504	3.507	3.471
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	13862.082	13128.206	13604.703	36349.089	36218.230	36140.695	2.622	2.759	2.656	2.679
Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	12390.900	12838.380	12768.194	37943.590	37427.189	37515.477	3.062	2.915	2.938	2.972
Regulation Factor and RNA Processing										
EF-Tu	50542.047	50463.220	50923.887	11632.601	11074.332	11369.138	0.230	0.219	0.223	0.224
elongation factor 1 beta	374768.567	370664.030	371835.340	112723.540	112922.180	112331.733	0.301	0.305	0.302	0.303

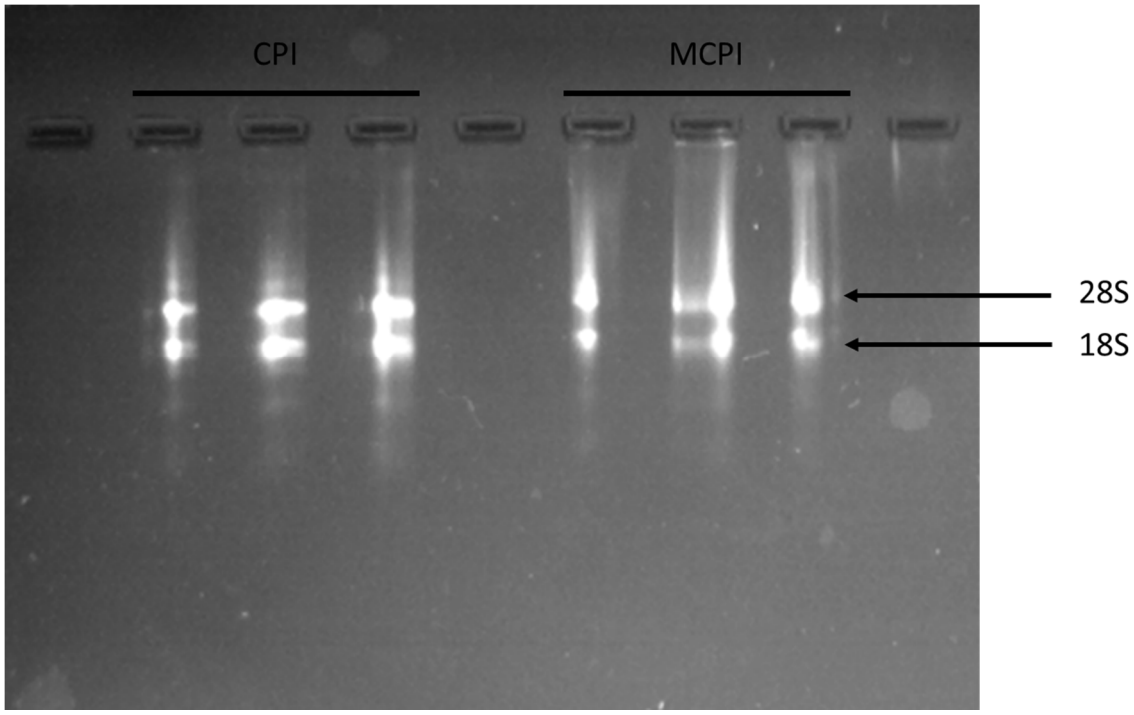
Supplementary Table 2.

Quantification and quality of total RNA extracted and purified from MCPI and CPI samples

Sample	ng/ul	260/280	260/230	Sample volume (µL)	Average ng/uL	ng Total
CPI R1	542.60	2.18	2.07	40	556.10	30244.00
CPI R1	570.00	2.18	2.13	40		
CPI R1	555.70	2.14	2.34	40		
CPI R2	550.00	2.23	2.13	40	555.90	22236.00
CPI R2	554.10	2.12	2.05	40		
CPI R2	563.60	2.16	2.18	40		
CPI R3	571.10	2.13	2.17	40	564.20	22568.00
CPI R3	557.30	2.13	2.16	40		
CPI R3	569.20	2.12	2.42	40		
MCPI R1	142.50	2.08	1.94	40	154.60	6184.00
MCPI R1	105.60	2.06	2.18	40		
MCPI R1	166.70	2.1	2.74	40		
MCPI R2	161.70	2.13	2.86	40	159.50	6380.00
MCPI R2	127.00	2.12	2.11	40		
MCPI R2	157.30	2.13	1.97	40		
MCPI R3	143.20	2.14	2.36	40	137.33	5493.33
MCPI R3	161.30	2.15	2.14	40		
MCPI R3	107.50	2.17	1.89	40		

Supplementary Figure 1.

RNA integrity of RNA extracted from CPI and MCPI



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CONSIDERAÇÕES FINAIS

Neste trabalho, foi demonstrado que o tratamento de sementes de feijão-de-corda susceptível à infecção pelo CPSMV, com o agente mutagênico EMS (0,04%) originou plantas mutagenizedes que apresentaram fenótipo de resistência (MCPI) quando desafiadas com o CPSMV. Os resultados obtidos com as plantas MCPI e CPI forneceram alguns pontos importantes sobre interações de resistência (MCPI) e susceptibilidade (CPI) do patossistema feijão-de-corda x CPSMV.

Durante a interação de incompatibilidade (MCPI), foi demonstrado a importância do envolvimento de diversos processos do metabolismo trabalhando de forma conjunta para conferir resistência a planta frente a infecção pelo CPSMV. A manutenção da alta taxa fotossintética, mesmo após a infecção, fornecendo suporte para demanda energética exigida durante as respostas bioquímicas clássicas envolvendo a indução de enzimas do metabolismo primário e secundário bem como o aumento das PR-proteínas.

Por outro lado, na análise da interação de compatibilidade (CPI), foi perceptível a indução, por parte do CPSMV, de alterações na síntese de proteínas do feijão-de-corda envolvidas com os mecanismos de defesa da planta. De fato, houve aumento na abundância de enzimas tais como CAT e APX, prevenindo o acúmulo de H_2O_2 e, portanto, a PCD, que é desfavorável ao vírus, e aumento na abundância de β-1,3-Glucanase, que degrada calose, resultando no aumento do tamanho de exclusão dos plasmodesmatas, favorecendo, respectivamente, o estabelecimento da infecção viral local e permitindo seu movimento célula a célula, para o posterior estabelecimento da infecção sistêmica.

Esses achados apresentam grande relevância por contribuírem para melhor se compreender como os principais eventos fisiológicos e bioquímicos estão envolvidos na interação de incompatibilidade (resistência) e compatibilidade (susceptibilidade) entre o feijão-de-corda e CPSMV, provendo subsídios para embasar melhor as respostas para as perguntas de partida do presente trabalho. Ao mesmo tempo, suscitam novas perguntas que, certamente, nortearão abordagens experimentais adicionais, que deverão, também, contribuir para um melhor entendimento das relações desse patossistema.