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RAISSA SOUZA CAMINHA BRET

METABOLIC CHANGES IN LOCAL AND SYSTEMIC COWPEA [*Vigna unguiculata* (L.) Walp.] LEAVES INDUCED BY COWPEA SEVERE MOSAIC VIRUS INFECTION

FORTALEZA
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Tese apresentada ao programa de Pós-Graduação em Bioquímica, do Centro de Ciências da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Doutora em Bioquímica. Área de Concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Danilo de Menezes Daloso.

Coorientador: Prof. Dr. Murilo Siqueira Alves.

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RESUMO

A suscetibilidade das plantas a doenças é um fenômeno complexo e regulado por mecanismos específicos de reconhecimento do patógeno e respostas de sinalização dentro da célula vegetal, que culminam em uma interação compatível ou incompatível entre o patógeno e a planta. Apesar da importância em entender os mecanismos pelos quais as plantas podem tolerar o ataque de um patógeno, o conhecimento dos fatores genéticos e bioquímicos que estão envolvidos na interação planta-patógeno ainda é escasso. Além disso, embora os mecanismos moleculares de percepção do vírus estejam relativamente bem documentados, as respostas metabólicas à infecção viral permanecem pouco compreendidas, especialmente no feijão-de-corda (*Vigna unguiculata* (L.) Walp.), no qual as respostas metabólicas à infecção viral não foram investigadas ainda. O feijão-de-corda é um importante alimento básico para regiões secas, mas a infecção por vírus pode diminuir a produção em até 85%, representando um grande gargalo para o cultivo do feijão-de-corda. Aqui, objetivamos investigar as respostas metabólicas dos genótipos de feijão-de-corda macaibo e pitiúba à infecção causada pelo vírus do mosaico severo do feijão-de-corda (CPSMV). Esses genótipos foram previamente caracterizados como resistente (macaibo) e susceptível (pitiúba) ao CPSMV. Uma extensa caracterização metabólica foi realizada usando folhas diretamente infectadas com CPSMV (folhas locais) e aquelas do mesmo trifolíolo (folhas sistêmicas), com o objetivo de identificar como a infecção por CPSMV influencia o metabolismo de folhas diretamente infectadas e adjacentes ao local da infecção. Análises metabolômicas foram realizadas usando cromatografia gasosa e líquida acoplada a espectrômetros de massa (GC-MS, LC-MS) para análise de perfil de metabólitos de metabólitos primários e análise de *fingerprinting* metabólica de metabólitos secundários. Nossos resultados mostraram que o metabolismo das folhas locais é substancialmente alterado pela infecção por CPSMV no nível do metabolismo primário, mesmo no genótipo resistente (macaibo). Notavelmente, a infecção por CPSMV alterou a dinâmica dos metabólitos primários nas folhas de macaibo, o que resulta em uma nova homeostase metabólica, mas mantém a estabilidade tanto da rede metabólica primária quanto do nível de metabólitos secundários. Além disso, mostramos que o metabolismo das folhas de macaibo e pitiúba difere substancialmente mesmo na ausência de infecção por CPSMV. Além de fornecer informações importantes sem precedentes sobre as respostas metabólicas de plantas de feijão-de-corda à infecção por CPSMV, nossos resultados destacam coletivamente que as respostas metabólicas à infecção por CPSMV diferem substancialmente entre genótipos de feijão-de-corda resistente e suscetível, nos quais um maior fluxo metabólico constitutivo em direção ao shikimato é observado em macaibo, quando comparado à pitiúba, e um menor fluxo metabólico do piruvato para o ciclo do TCA é observado na infecção por CPMSV em macaibo.

Palavras-chave: marcação ^{13}C; *Vigna unguiculata*; CPSMV; metabolômica; shikimato.

ABSTRACT

The susceptibility of plants to diseases is a complex phenomenon and regulated by specific mechanisms for recognition of the pathogen and signalling responses within the cell, which culminate in a compatible or incompatible interaction between the pathogen and the plant. Despite the importance in understanding the mechanisms by which plants can tolerate a pathogen attack, the knowledge of the genetic and biochemical factors that are involved in the plant-pathogen interaction is still scarce. Furthermore, although the molecular mechanisms for virus perception are relatively well-documented, the metabolic responses to viral infection remain poorly understood, especially in cowpea (*Vigna unguiculata* (L.) Walp.) in which the metabolic responses to virus infection has not been investigated yet. Cowpea is an important staple food for dry regions, but virus infection can decrease yield by up to 85%, representing a major bottleneck for cowpea cultivation. Here, we aimed to investigate the metabolic responses of macaibo (resistant) and pitiúba (susceptible) cowpea genotypes to infection caused by cowpea severe mosaic virus (CPSMV). An extensive metabolic characterization was performed using leaves directly infected with CPSMV (local leaves) and those from the same trifoliate (systemic leaves), aiming to identify how CPSMV infection influences the metabolism of directly infected leaves and those adjacent to the site of infection. Metabolomics analyses were performed using gas and liquid chromatography coupled to mass spectrometers (GC-MS, LC-MS) for metabolite profiling analysis of primary metabolites and metabolic fingerprinting analysis of secondary metabolites, respectively. Our results showed that the metabolism of local leaves is substantially altered by CPSMV infection at primary metabolism level, even in the resistant (macaibo) genotype. Notably, CPSMV infection altered the dynamic of primary metabolites in macaibo leaves, which results in a new metabolic homeostasis but maintain the stability of both the primary metabolic network and the level of secondary metabolites. We further showed that the metabolism of macaibo and pitiúba leaves differ substantially even in the absence of CPSMV infection. Beyond providing unprecedented important information on the metabolic responses of cowpea plants to CPSMV infection, our results collectively highlight that the metabolic responses to CPSMV infection differ substantially between resistant and susceptible cowpea genotypes, in which a higher constitutive metabolic flux toward shikimate is observed in macaibo, when compared to pitiúba, and a lower metabolic fluxes from pyruvate to the TCA cycle is observed upon CPMSV infection in macaibo

Keywords: ^{13}C-labelling analysis; *Vigna unguiculata*; CPSMV; metabolomics; shikimate.

CONTENT

1 INTRODUCTION

Phytopathogenic viruses are currently one of the major sources of yield loss for a wide range of crop plants. Given that it is not possible to overcome virus infection by agrochemicals, as it is performed in a large number of plant-pathogen interactions, the only manner to struggle many of the viruses is by prophylactic mechanisms or by creating transgenic plants tolerant to such pathogens (NICAISE, 2014). In the state of Ceará, Brazil, the cowpea severe mosaic virus (CPSMV) is the main cause of yield losses in cowpea (*Vigna unguiculata* (L.) Walp.), an important crop for dry regions worldwide, including small and medium farmers of Ceará (SILVA et al., 2016). Unfortunately, cowpea genotypes resistant to CPSMV generally have low yield capacity, which limits their use (PAIVA et al., 2014). This is explained by the growth-stress tolerance trade-off, in which plants with higher capacity to grow are commonly more susceptible to stress, and *vice-versa* (LIU et al., 2019). Understanding the mechanisms that regulate this trade-off is thus crucial to maintain cowpea yield and obtain stress tolerant cowpea genotypes through metabolic engineer (CORREA et al., 2020).

Contrary to the popular belief, plants are able to communicate their different parts through signalling compounds as well as with other plants by electrical signalling or volatiles in response to endogenous and/or environmental signals (BROSSET; BLANDE, 2022; NGOU; DING; JONES, 2022; TABASSUM; BLILOU, 2022). Plants have several mechanisms for defence, still in debate whether it can be considered an immune system. The action and responsiveness of several defence mechanisms such as PAMP-triggered immunity (PTI), effector-triggered immunity (ETI), systemic acquired response (SAR), and hypersensitive response (HR) have been relatively well-studied (CHANG et al., 2022; ERICKSON et al., 2022). Furthermore, several signalling pathways activated after pathogen recognition have already been described, in which hormones and secondary metabolites have been exhaustively studied and associated with defence mechanisms. However, although the role of primary metabolites for plant abiotic stress acclimation has been unveiled in the last decades using diverse metabolomics approaches (FÀBREGAS; FERNIE, 2019), how this metabolism influence the defence against biotic stresses is much less investigated.

Primary and secondary metabolisms have been generally studied as distinct and independent pathways. This is especially due the fact that primary and secondary metabolisms are mostly associated to growth and defence, respectively (SOUZA et al., 2020). However, plant metabolism operates as a highly integrated network, in which primary and secondary

metabolisms are interconnected and dependent on each other (SHACHAR-HILL, 2013). Furthermore, recent evidences suggest that primary metabolites are key for plant acclimation to biotic and abiotic stress conditions (ZANDALINAS et al., 2022). Notably, glutamate, which has been traditionally investigated in association to its function in the nitrogen assimilation in the primary metabolism, recent evidences suggest that this metabolite also has an important role for defence, especially in the communication between organs upon pathogen attacks (HORNYÁK et al., 2022). However, despite the increasing number of studies associating primary metabolism with plant acclimation to abiotic stresses, plant metabolic responses to virus infection are less understood and seem to be pathosystem dependent (LLAVE, 2016). This preconizes that the current available information (i) cannot be used for a different pathosystem and (ii) it is insufficient to guide plant metabolic engineer to obtain stress tolerant genotypes, highlighting the need for further studies. This is severer for the cowpea-CPSMV pathosystem, in which no information on the role of primary and secondary metabolites for CPSMV resistance is available. It is clear therefore that studies aiming to better understand how CPSMV infection alter the metabolism of cowpea genotypes are needed. Taking this into account, we have carried out an extensive metabolic characterization of resistant (macaibo) and susceptible (pitiúba) cowpea genotypes under CPSMV infection using different state-of-art metabolomics approaches.

Immune Nonhost		Virus does not replicate in protoplasts or in the initially inoculated cells of the intact plant. Inoculum virus may be uncoated, but no progeny viral genomes are produced.
Infectible host	Resistant (extreme hypersensitivity)	Virus multiplication is limited to initially infected cells because of an ineffectual virus-coded movement protein, giving rise to subliminal infection. Plants are field resistant.
	Resistant (hypersensitivity)	Infection limited by a host response to a zone of cells around the initially infected cell, usually with the formation of visible necrotic local lesions. Plants are field resistant.
	Susceptible	Systemic movement and replication.
	Sensitive	Plants react with more or less severe disease.
	Tolerant	There is little or no apparent effect on the plant, giving rise to latent infection.

Table 1. Plant classification according to their responses to virus inoculation. (HULL, 2009)

Accordingly to Hull (2009), different plant-virus interactions culminate in a different compatibility (Table 1). Until today, macaibo was considered immune to CPSMV

infection (LIMA et al., 2011; LIMA et al., 2012), once it was not identified CPSMV presence or host response at the time. This is the first study to provide actual evidence that macaibo displays metabolic response in order to suppress CPSMV infection and virus detection through PCR. Herein, macaibo is now classified as resistant to CPSMV infection.

The thesis is presented in two chapters. The first involves the characterization of the metabolic responses of macaibo (highly resistant to CPSMV infection) leaves directly infected with CPSMV (local leaves) and those from the same trifoliate (systemic leaves). For this, we have used two metabolomics platforms, namely gas and liquid chromatography's coupled to mass spectrometers (GC-MS, LC-MS), which were used to identify metabolites pertaining to primary and secondary metabolisms, respectively. The results of this chapter showed that CPSMV infection induced changes in primary metabolism of both local and systemic macaibo leaves, but the response to CPSMV was different among them and no alteration was found in secondary metabolism. Meanwhile, the second chapter involved the characterization of both macaibo and pitiúba (highly susceptible to CPSMV infection) leaves to CPSMV infection, aiming to compare the primary metabolism of these genotypes under CPSMV infection. For this, we have used two GC-MS-based metabolomics approaches, that is metabolite profiling and ^{13}C-labelling analysis. The results showed that macaibo and pitiúba leaves have distinct metabolite profiles even in the absence of CPSMV infection. Additionally, a higher metabolic flux toward shikimate was detected in macaibo, when compared to pitiúba leaves, despite the presence/absence of CPSMV. Likewise, CPSMV infection reduced the metabolic fluxes from pyruvate to the tricarboxylic acid (TCA) cycle in macaibo, and this was not observed in pitiúba. Our results collectively provide unprecedented information concerning the cowpea metabolic responses to CPSMV infection. We unveiled that macaibo responses to CPSMV infection involve changes in primary metabolism, which were contributed to handoff virus infection, maintaining the stability of the primary metabolic network and the level of secondary metabolites in this genotype.

1.1 Hypothesis

The resistance of macaibo to CPSMV infection is associated to changes in the level of primary and secondary metabolites that aid these plants to struggle virus infection, while the differential tolerance to CPSMV between macaibo and pitiúba is also associated to their differences at primary metabolism level

1.2 Objectives

1.2.1 General

To investigate leaf cowpea's metabolic responses to CPSMV infection of two extremes level of susceptibility phenotypes.

1.2.2 Specific

i To identify metabolites that are responsive to CPSMV infection;
ii To analyse the similarities and discrepancies between the metabolic responses of local and systemic leaves to CPSMV infection;
iii To investigate whether the dynamic of accumulation of primary metabolites and the topology of the metabolic network is altered by CPSMV infection;
iv To explore whether the secondary metabolism is affected by CPSMV infection;
v To compare the metabolic responses to CPSMV infection between resistant and susceptible cowpea genotypes;
vi To examine the effect of CPSMV infection on the ^{13}C-distribution derived from glucose throughout the primary metabolism.

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2 CHAPTER I: COWPEA SEVERE MOSAIC VIRUS (CPSMV) INFECTION ALTERS THE DYNAMIC OF LEAF PRIMARY METABOLISM BUT HAS LITTLE IMPACT ON THE SECONDARY METABOLISM OF A CPSMV-RESISTANT GENOTYPE

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Running tittle: The cowpea metabolic responses to virus infection

Highlight: CPSMV infection altered the dynamic of primary metabolites, especially branched chain amino acids, but had little impact on the secondary metabolism and the topology of the primary metabolic network

2.1 Abstract

Plant virus interactions depends on an intricate battle that culminates in a compatible or incompatible interaction between them. Whilst the signalling pathways downstream virus infection are well-described, local and systemic metabolic responses to virus infection remain to be unveiled. Here, we carried out a time-series metabolic characterization of leaves directly infected with cowpea severe mosaic virus (CPSMV) (local leaves) and those of the same leaflet (systemic leaves) using a CPSMV-resistant cowpea genotype. PCR analysis of the CPSMV coat protein indicates that viral infection increased in the first 8 h of virus inoculation, but it decreased in the following three days. Multivariate analysis clearly distinguished virus-infected local leaves from both non-infected local and virus-infected systemic leaves at primary metabolism level. However, no substantial changes at secondary metabolism level were observed. The dynamic of accumulation/degradation of primary metabolites, especially branched chain amino acids, was altered throughout virus infection time. However, the density and heterogeneity of the metabolic network remained relatively stable in local-infected leaves. Our results highlight that CPSMV infection substantially alters the dynamic and accumulation of primary metabolites, which results in a new metabolic homeostasis and do not disturb the stability of both the primary metabolic network and the level of secondary metabolites.

Key words: metabolic fingerprinting, metabolic network, metabolite profiling, plant-virus interaction, systems biology.

2.2 Introduction

Plants are constantly subjected to adverse conditions, which require local and systemic responses to acclimate to the prevailing surrounding environmental condition (ZANDALINAS et al., 2020). Given the current climate change scenario, in which (a)biotic stress periods are predicted to be more frequent and intense (ZANDALINAS et al., 2021), it is crucial to find strategies to improve crop yield and/or stress resilience in crops (EVANS; LAWSON, 2020). However, there is a clear yield-stress tolerance trade-off in plants, in which stress tolerance is generally achieved at expenses of productivity, and *vice-versa* (CÂNDIDO-SOBRINHO et al., 2022; FERNANDEZ et al., 2021). For instance, the model of our study, namely the cowpea (*Vigna unguiculata* (L.) Walp.) Macaibo genotype, is resistant to cowpea severe mosaic virus (CPSMV) but has much lower yield than other sentive and high productive cowpea genotypes (LIMA et al., 2011; PAIVA et al., 2014). Cowpea is a leguminous crop that is very important for regions with dry soil, thanks to its low-cost production, high nutritional value and high tolerance to conditions of drought (KAREEM; TAIWO, 2007; SINGH et al., 2011). However, infection by CPSMV, a Comovirus (*Secoviridae*) that infects *Fabaceae* plants, can reduce yield by up to 85%, representing a major bottleneck for cowpea cultivation (BOOKER; UMAHARAN; MCDAVID, 2005; KAREEM; TAIWO, 2007). Understanding the mechanisms by which cowpea plants respond to CPSMV is thus important to find strategies to maintain the cultivation and productivity of this important staple food.

Plant virus interactions are characterized by compatible and non-compatible interactions. The compatible interaction results in strong virus infection in a sensitive plant genotype, while incompatible interaction is defined by the absence of symptoms caused by virus infection in the hosted plant (HULL, 2013). The severe mosaic, disease caused by CPSMV, presents clear symptoms such as yellowish mosaic-like spots on leaves and, in more advanced infection cases, may leads to plant dwarfism and cell death spots on leaves (LALIBERTÉ; ZHENG, 2014; RYS et al., 2014). CPSMV is an obligatorily intracellular biotrophic pathogen, meaning that it cannot be controlled by prophylactic or pesticide treatments, which makes its control difficult in a large scale cultivation (HULL, 2009; NICAISE, 2014). Furthermore, the resistance to CPSMV is polygenic, which hampers the development of resistant cultivars (YOUNG, 2000). In the CPSMV-Macaibo incompatible interaction, no severe symptoms appear in Macaibo leaves after CPSMV infection (LIMA et al., 2011; MAGALHÃES, 2011). Macaibo, therefore, represents an important genetic

resource to understand the mechanisms of plant-virus interaction, which is crucial to obtain not only virus-resistant cowpea but also other virus-resistant *Fabaceae* genotypes.

Previous study has demonstrated that the CPSMV-resistant BRS-Marataoã cowpea genotype displays no morphological symptoms 6 days after CPSMV infection. However, significant alterations in the level of compounds and activity of enzymes related to the redox metabolism were observed, highlighting that CPSMV resistance involves changes at biochemical level (VARELA et al., 2017). Furthermore, proteomic analysis of this incompatible interaction unveiled that proteins related to both energy and carbohydrate metabolisms were among the main functional categories altered by CPSMV infection (VARELA et al., 2017). These results suggest that the cowpea tolerance to CPSMV involves changes in primary metabolism. By contrast, studies with the susceptible cowpea genotype CE-31 highlight that CPSMV infection increases the content of proteins related to redox homeostasis, defence and energetic metabolism (MAGALHÃES 2011; PAIVA, et al. 2016; SILVA et al. 2016). However, certain basic information concerning virus defence mechanisms remains unavailable for this pathosystem. For instance, how systemic acquired resistance (SAR) is established, and which compounds act as communicators between infected and non-infected sites of the plant are still not understood.

Although it is much reported that secondary metabolites such as alkaloids, terpenes and phenolic compounds play a key role as plant chemical defensives against pathogens, very little is known about the role of primary metabolites in the battle against viruses (ISLAM, et al. 2019). This is especially true for the Cowpea-CPSMV pathosystem. Among the metabolic pathways activated following viral entrance, it seems that the activation of the mitochondrial respiratory metabolism is important for plant defence in both compatible and incompatible pathosystems (DI CARLI et al., 2010; LLAVE, 2016). However, the changes in the metabolism of carbohydrates and amino acids range according to the pathosystem (ISLAM et al., 2019; LESS et al., 2011; LLAVE, 2016). For instance, virus infection leads to increased sugar content in *Cucumber* and *Tobacco*, whilst decreased sugar content in both *Potato* and *Turnip yellow* after virus infection was observed (FERNÁNDEZ-CALVINO et al., 2014; SADE et al., 2015). These results suggest that the function of primary metabolites involved in plant defence and/or establishment of viral infection is pathosystem-dependent and cannot be generalized based on a model pathosystem (ISLAM et al., 2019; LESS et al., 2011; LLAVE, 2016). It is clear therefore that several pieces of the cowpea-CPSMV puzzle are yet to be discovered.

Recent evidence based in biosensor analyses highlights that both local biotic and abiotic stresses stimulus trigger rapid and systemic responses throughout the other parts of the plant (DEVIREDDY; ARBOGAST; MITTLER, 2020; FICHMAN; MITTLER, 2021; JR; FICHMAN; MITTLER, 2022; ZANDALINAS et al., 2020). These studies have demonstrated that stress conditions are propagated throughout the plant via waves of Ca^{2+}, ROS and other mobile molecules. Furthermore, it seems that waves of Ca^{2+} and electrical signals used to communicate and activate systemic responses in plant organs that have not directly received the stress are dependent on the glutamate receptor GLR (CAI; AHARONI, 2022; FENG et al., 2021; GRENZI; BONZA; COSTA, 2022). GLR is also known to be involved in priming associated to biotic stress and pattern-trigged immunity (PTI) activation (CAI; AHARONI, 2022; GRENZI; BONZA; COSTA, 2022). This suggests that plant metabolism is likely involved in the coordination of local and systemic stress responses. Despite the fact that classic mechanisms of plant defence such as PTI, effector-triggered immunity (ETI), SAR and induced systemic resistance (ISR) are well described for different pathosystems, the metabolic signatures of local and systemic responses remain to be unveiled, especially in response to virus infection. We thus aimed to unravel the metabolic responses of local and systemic leaves of the virus-resistant Cowpea Macaibo genotype during the progression of CPSMV infection. We carried out an extensive metabolic characterization of infected and non-infected local and systemic leaves. Our results unveiled that CPSMV resistance is achieved through alteration in the dynamic of primary metabolism, but with little impact on the secondary metabolism and in the topology and connectivity of the primary metabolic network.

2.3 Material and Methods

2.3.1 Plant material and growth conditions

We used the Cowpea (*Vigna unguiculata* (L.) Walp.) Macaibo genotype, which is resistant to CPSMV infection. Macaibo seeds were disinfected by adding sodium hypochlorite. Seeds were washed in distilled water and germinated in filter paper rolls (28x38 cm; Germitest) soaked with distilled water, sealed in clear plastic bags and kept in a growth chamber for 7 days with a 12 h photoperiod, 50 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The seedlings were transferred to 6 L plastic pots (6 seedlings per pot) and maintained under hydroponic system using Hoagland and Arnon's nutrient solution with slight modifications (HOAGLAND; ARNON, 1950; SILVEIRA; COSTA; OLIVEIRA, 2001).

Plants were kept under greenhouse conditions with natural sunlight (300-650 µmoles m^{-2} s^{-1} PPFD), with daily temperature ranging from 27 to 31.0 °C and 79.8 ± 10.9% of average relative humidity.

2.3.2 Isolation of CPSMV

The inoculum of Cowpea severe mosaic virus (CPSMV), strain CE, was isolated from previously infected leaves of Cowpea, genotype CE-31, as described previously (PAZ et al., 1999). Virus-infected leaves of Cowpea CE-31 were macerated in 10 mM potassium phosphate buffer, pH 7.0, containing sodium sulfite (1:10, m/v) for 10 minutes. Carborudum (600 mesh) was added to the inoculum (1:10, m/v) to facilitate infection in the leaf tissues.

2.3.3 Inoculation of CPSMV into Cowpea leaves

Cowpea leaves are disposed as trifoliate, composed by a central leaf surrounded by two lateral leaves (Figure 1A). The inoculation of Mock and CPSMV were performed on the central leaf of the first fully expanded trifoliate by friction with thumb and index fingers. Control plants were Mock inoculated with same solution, however without CPSMV added (Figure 1A). Central (called herein as local leaf) and lateral (called herein as systemic leaf) leaves of cowpea Macaibo were harvested after 02, 04, 08, 24, 48 and 72 hours after virus or mock inoculation (HAI) and frozen in liquid nitrogen for metabolomics analysis.

2.3.4 Virus detection in cowpea leaves

Viral infection was assessed on infected Cowpea leaves by PCR analysis. Frozen Cowpea leaves were macerated with liquid nitrogen until a fine powder. Total RNA was extracted using SV Total RNA Isolation System kit (Promega) according to the manufacturer's instructions. The quantity of RNAs was quantified using spectrophotometer (BioTek Instruments) and their integrity were evaluated by 1.0% agarose gel electrophoresis with ethidium bromide (0.5 µg mL^{-1}). The synthesis of cDNA was performed using M-MLV Reverse Transcriptase system (Promega) according to the manufacturer's instructions. A portion of the CPSMV coat transcript (cpCPSMV KF793280) was amplified through PCR by GoTaq DNA Polymerase (Promega), 300 nmol of each primer (FW: GCA TGG TCC ACW AGG T and RV: YTC RAA WCC VYT RTT KGG MCCACA) and 100 µg of cDNA were

added to the reaction mixture. The amplicon was detected in 1.0% agarose gel electrophoresis with ethidium bromide (0.5 µg mL^{-1}).

The CDS region of the CPSMV viral movement protein was selected from its genome, which is available in the NCBI genomic database (https://www.ncbi.nlm.nih.gov/genome/) (CHEN; BRUENING, 1992a; CHEN; BRUENING, 1992b). After identifying the selected CDS regions, they were used as templates for constructing primer pairs using Perl Primer v1.1.19 software for amplification via conventional PCR (PCR) or real-time quantitative PCR (RT-qPCR). The constructed primer pairs will have their efficiency and annealing temperature tested as proposed by Livak and Schmittgen (2001).

2.3.5 GC-TOF/MS-based metabolite profiling analysis

The extraction of polar metabolites and the GC-TOF/MS analysis was carried out as described previously (LISEC et al., 2006). Samples were macerated with liquid nitrogen until a fine powder. 50 mg was then transferred to a microtube (2.0 mL), where 1.4 ml of methanol and 60 µL of ribitol (0.2 mg/mL in deionized water) were added. The following steps were conducted under dark conditions. Microtubes were shaken (950 r.p.m.) for 15 min at 37 ºC, followed by centrifugation (11,000 *g*) for 10 min. The supernatant was transferred to a new microtube (2.0 mL), where 750 µL of cold chloroform and 1.4 µL of deionized water were added. After mixing by vortex and centrifugation (10,000 *g*, 15 min), aliquots of 150 µL from upper (polar) phase were taken and dried using speed vac. Subsequently, samples were derivatized by adding 40 µL of methoxyamine hydrochloride, previously dissolved in 20 mg/mL in pure pyridine and shaken at 37 ºC for 2 h. We next added 70 uL of *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and tubes were shaken again at 37 ºC for further 30 min. After that, a centrifugation was performed, and the supernatant was transferred to vials and injected in GC-TOF/MS. Analysis of the mass chromatograms was carried out using the Xcalibur 2.1 software (Thermo Fisher Scientific). Metabolites were identified using the Golm Metabolome Database (KOPKA et al., 2005).

2.3.6 LC/MS analysis

Ultra-high-performance liquid chromatography high-resolution mass spectrometry (LC/MS) analysis was carried out as described earlier (TOHG; FERNIE, 2010),

using the same leaf material used for GC-TOF/MS. This LC/MS platform mostly detect medium polarity secondary metabolites (PEREZ DE SOUZA et al., 2021). We first used a *metabolic fingerprinting* approach, which provides a global overview of the features detected by LC/MS without the need for metabolite identification (PEREZ DE SOUZA et al., 2019). This analysis was carried out by using 13.586 features (peaks found in the chromatograms). After that, the metabolites were annotated by using MZmine (PLUSKAL et al., 2010), which resulted in 98 metabolites identified.

2.3.7 Metabolic network analysis

Debiased sparse partial correlation (DSPC) were calculated among the metabolites of the metabolic networks using Lasso Penalty Method and the CorrelationCalculator software. Correlation-based metabolic networks were build using MetScape on Cytoscape software considering $-0.5 > r > 0.5$. The parameters density and heterogeneity of the metabolic networks were obtained as described earlier (ASSENOV et al., 2008). We further calculated and identified the number of hubs of the network as described earlier (FREIRE et al., 2021). Briefly, a node was considered a hub when its degree of connection (number of links) was higher than the average of the degree of connection of the network in a given mock-treated leaf and time point. For example, if the mean degree of connection of the mock-treated local leaf at 02 HAI is 3, all nodes with degree of connection higher than 3 at 02 HAI are considered hubs.

2.3.8 Experimental design and statistical analysis

Experiments were completely randomized in a factorial arrangement consisted of 2 treatments (mock and virus inoculation), 2 organs (local and systemic leaves) and 6 time points (02, 04, 08, 24, 48 and 72 hours after infection (HAI)). The metabolomics data was evaluated using analyse of variance (ANOVA) followed by the Tukey´s test ($P < 0.05$) and partial least squares-discriminant analysis (PLS-DA) using the MetaboAnalyst platform (CHONG et al., 2018). This data was also analysed using a time series approach named clustering dynamic time warping (dtw) through the TSclust on R software (MONTERO; VILAR, 2015). Heat maps were build using the MetaboAnalyst platform (using none sample normalization, log transformation as data transformation and auto scaling as data scaling) or

the MultiExperiment Viewer 4.9.0 software and manually integrated into a metabolic pathway.

2.4 Results

2.4.1 CPSMV do infect cowpea macaibo leaves, but no visual symptom is observed

The cowpea macaibo genotype has been characterized as resistant to CPSMV infection (LIMA et al., 2011). However, the metabolic basis underpinning such tolerance remains unclear. Here we infected cowpea macaibo leaves with CPSMV and performed an extensive metabolic characterization in leaves directly infected with CPSMV (herein called local leaves) and those leaves pertaining to the same trifoliate (herein called systemic leaves) (Figure 1A). No visual symptoms appeared after 15 days of CPSMV inoculation neither in local nor in systemic leaves (Figure 1A). However, PCR analysis of the CPSMV coat protein revealed an increase in the infection, especially in the first day of infection (from 2 to 8 Hours After CPSMV Inoculation (HAI)), as compared to the negative control (Mock). Interestingly, the PCR product of the CPSMV coat protein decreased from 8 to 24, 48 and 72 HAI, resembling more the negative than the positive (CPSMV) control (Figure 1B). These results indicate that CPSMV can infect cowpea macaibo leaves, but this genotype nearly eliminate the virus after the first day of infection. This explains the lack of symptoms after 15 days of CPSMV infection and strengthen the idea that Macaibo is highly resistant to CPSMV. This is also corroborated by Magalhães (2011), which shows that macaibo counterattacks CPSMV attempt to infection through biochemical enzymes. We then next decided to investigate what are the metabolic basis for this tolerance.

2.4.2 Leaf primary metabolism is altered in CPSMV-infected leaves

To have a clear picture of the cowpea Macaibo metabolic responses to CPSMV, we carried out an extensive metabolic characterization of local and systemic leaves inoculated with mock or CPSMV for 02, 04, 08, 24, 48 and 72 h. We first used a well-established gas chromatography *time-of-flight* mass spectrometry (GC-TOF/MS) platform that detects polar metabolites mostly from primary metabolism (LISEC et al., 2006). This analysis highlights that the primary metabolism was substantially modified upon CPSMV infection (Figure 2). 22 and 17 metabolites displayed a differential accumulation over time in local and systemic

leaves, respectively (Table 1). The level of 21 and 14 metabolites was significantly ($P < 0.05$) altered upon CPSMV infection in local and systemic leaves in at least one time point, when compared to their respective mock control, respectively (Table 1; Figure 2). Partial least squares-discriminant analysis (PLS-DA) clearly discriminates infected from non-infected local and systemic leaves, with a great extent to the local leaves, in which a clearer separation by the first component was observed (Figure 3). PLS-DA using the data of local and systemic CPSMV-infected leaves normalized according to their respective mock control further distinguished the metabolic responses of local and systemic leaves to CPSMV, as evidenced by the separation by the first components (Figure 4). This analysis provides a variable importance in projection (VIP) score for each parameter used in the PLS model. Metabolites with VIP score higher than 1 are those that most contributed to the separation found in the PLS-DA graph (XIA; WISHART, 2011). Citrate, nicotinic acid, Val, Leu and Ile have VIP score higher than 1 in both local and systemic leaves in at least one time point (Table S1). Punctual analysis highlights that these metabolites were significantly altered by CPSMV inoculation in local leaves at 48 HAI, with exception of the nicotinic acid. Among them, only citrate showed lower level in CPSMV-infected leaves, as compared to mock treated local leaves (Figure 5). Nicotinic acid, Val, Leu and Ile are also included in the VIP score list of the PLS model that discriminated local from systemic infected leaves (Table S1). Although no visual symptoms were observed in leaves infected with CPSMV, our GC-TOF/MS analysis indicates that the primary metabolism was substantially modified upon CPSMV infection.

2.4.3 Metabolic fingerprinting analysis highlights that leaf secondary metabolism is slightly affected by CPSMV inoculation

We next used a ultra-high-performance liquid chromatography high-resolution MS (LC/MS) platform dedicated to detecting mostly secondary metabolites (PEREZ DE SOUZA et al., 2021; TOHGE; FERNIE, 2010). We first carried out a metabolic fingerprinting analysis that do not require metabolite identification. This approach is useful to discriminate samples based in the intensity of the features (peaks) identified in the chromatograms (CÂNDIDO-SOBRINHO et al., 2022; KOSMIDES et al., 2013). Among the >13,000 features detected (Figures 6A-B), which mostly belong to the secondary metabolism according to a pathway enrichment analysis (Figure S1), none of them were statistical different over time and between treatments by ANOVA (Table S2A). When the data of CPSMV-infected leaves are normalized according to the values found in their respective

mock control, 34 and 35 features were found to display differential accumulation over time in local and systemic leaves, respectively (Table S2B). However, only one feature was significantly different between mock and CPSMV treatments in systemic leaves at 08 HAI (Table S2C), indicating that the secondary metabolism was not highly affected by CPSMV inoculation in the period analysed here. We next identified 98 metabolites from the LC/MS data (Figures S2A-B). None of these 98 metabolites was significantly different overt time and between the treatments as assessed by ANOVA (Table S3). When the data from CPSMV-infected leaves are normalized according to the values found in mock-treated leaves, only one and two metabolites changed over time in systemic and local leaves, respectively (Table S3). Our metabolomics analyses collectively suggest that the major metabolic changes induced by CPSMV infection are observed at the level of primary rather than secondary metabolism. We then next deeply explored the GC-TOF/MS data using other systemic analyses.

2.4.4 The dynamic of accumulation/degradation of primary metabolites is altered in CPSMV-infected leaves

Plant metabolism is highly dynamic, and its regulation ranges in both space (i.e. according to the organ/tissue) and time (SWEETLOVE; FERNIE, 2013). To account for the latter, we next adopted a time-series approach based on dynamic time warping (DTW) and hierarchical clustering to better investigate the changes in primary metabolites in local and systemic leaves over time of CPSMV infection. DTW analysis cluster metabolites with similar dynamic over time using a maximum-minimum transformation, i.e. the level of the metabolites is transformed to a 0 (lowest average value) to 1 (highest average value) scale (CÂNDIDO-SOBRINHO et al., 2022; DEANS et al., 2019). Twenty clusters were generated including data from non-infected and infected local and systemic leaves (Figure S3). This analysis unveiled that 22 and 14 metabolites have changed their pattern of accumulation/degradation in local and systemic leaves over time under CPSMV, respectively (Table S4). Interestingly, Leu from local and systemic mock-treated leaves was found in the clusters 5 and 16, respectively, but have the dynamic of the cluster 3 in both local and systemic leaves after CPSMV infection (Figures 7A,C). By contrast, Ala from non-infected local and systemic leaves was found in the cluster 7 and then in the clusters 13 and 3 in local and systemic leaves after CPSMV infection (Figure 7B,D). Similar differences were also observed in certain sugars (glucose, sucrose), organic acids (malate and succinate), amino acids (Pro, Ser, and homoserine), and GABA, glycerate and glycolate, especially in local

leaves (Figures 7A-D). Stress-related metabolites such as nicotinic acid and salicylic acid have been grouped in different clusters in CPSMV-infected local leaves, whilst only salicylic acid was grouped in a different cluster after virus infection in systemic leaves (Table S4). This analysis highlights that the dynamic of primary metabolites is substantially altered by CPSMV infection.

2.4.5 The density and the topology of the leaf primary metabolic network is relatively unaffected by CPSMV infection

We have recently shown that multivariate coupled with correlation-based metabolic network analyses aid to unveiling emergent properties of plant responses to stress conditions (CARDOSO; FREIRE; DALOSO, 2022). We then next applied a correlation-based network approach to investigate the changes in both density and topology of leaf metabolic networks induced by CPSMV infection. Nodes and links correspond to the metabolites identified by GC-TOF/MS and the debiased sparse partial correlation (DSPC) among them, respectively (FREIRE et al., 2021). Metabolic networks created combining all GC-TOF/MS data revealed that CPSMV infection increased and decreased the density and the heterogeneity of the networks, respectively, in both local and systemic leaves (Figures 8A-B). Interestingly, Ile, Leu and Val appear as a module highly connected to each other in CPSMV-infected local leaves (Figure 8A). This is even more evident when the links of the networks are restricted to the significant correlations ($P < 0.05$), in which an isolated, highly connected module (subnetwork) composed by Ile, Leu, Val and homoserine is formed (Figures 9A-B).

These network analyses suggest that CPSMV infection leads to metabolic networks more connected (higher density) and with the presence of more hubs (lower heterogeneity). However, when the networks were analysed in each time point (Figures S4-S5), no clear pattern of changes in either density or heterogeneity of the networks were observed in CPSMV-infected leaves (Figures 10A-D). For instance, whilst the density of mock-treated local leaves reduced over time, this parameter oscillated from 2 to 72 HAI in CPSMV-infected local leaves (Figure 10A). This result highlights that, although the primary metabolism is substantially altered in CPSMV-infected local leaves, the structure of the metabolic network is relatively unaffected.

2.4.6 Branched-chain amino acids are the major hubs of CPSMV-infected leaf metabolic networks

We next identified the hubs of these networks and investigated their behaviour throughout the time of CPSMV infection. Interestingly, the number of hubs and the number of exclusive hubs was substantially higher in mock-treated local leaves, when compared to CPSMV-treated local leaves (Figures 10E,G). In the systemic leaves, these parameters oscillated in mock and CPSMV-treated leaves over time, with exception of the number of hubs in mock-treated systemic leaves that remained relatively stable over time (Figures 10F,H). Among the hubs identified in the different time points, nicotinic acid is the one that most appeared in the different time points in CPSMV-infected local leaves, followed by glucoheptose and Gln (Figure S6). Several metabolites related to the TCA cycle such as fumarate, pyruvate, oxalate, malate, malonate, succinate, and citrate were found as hubs in CPSMV-infected local leaves. Glucose, sucrose and fructose also appeared as hubs in CPSMV-infected local leaves. Interestingly, the branched-chain amino acids (BCAA) Ile, Leu and Val were identified as hubs after 72 HAI in local leaves (Figure S6). Similarly, Ile and nicotinic acid at 48 HAI and Val at 72 HAI were also found as hubs in CPSMV-infected systemic leaves (Figure S7).

Comparing local and systemic infected leaves (Figure S8), it is clear that CPSMV presence modified the metabolic network of cowpea differently in local and systemic leaves. Even though a few important hubs (e.g. glucose at 4 HAI, lactate at 24 HAI, and nicotinic acid at 48 HAI) were found as hubs in both types of leaves, the number of hubs range substantially between local and systemic leaves over time (Figures 8E-F). Ile appear as hub in all time points in local leaves, whilst Val appears at 2, 4, 24, 48 and 72 HAI and Leu appears at 4, 8 and 48 HAI. Homoserine, Thr, butyrate, and nicotinic acid appeared as hubs only in local leaves. Ile, glycolate, Gly, Val and *myo*-inositol appeared as hubs in systemic leaves in different time points (Figure S9). These results, coupled to the other analysis, suggest that nicotinic acid and BCAAs are prominent hubs that underpin the CPSMV resistance in cowpea leaves.

2.5 Discussion

2.5.1 The power of metabolomics in unveiling silent phenotypes

Plant stress acclimation can involve changes in different levels, from the morphological to the epigenetic. Stress-induced responses are highly integrated between the different modules of the plant system (i.e. molecular, biochemical, physiological, morphological etc). However, these modules operate at different temporal scales (GALVIZ; SOUZA; LÜTTGE, 2022). This implicates that the activation of certain modules is faster than others under stress. For instance, the metabolic responses to oxidative stress are much faster than the changes in gene expression (LEHMANN et al., 2009), which partially explain the lack of correlation between metabolomics and gene expression data (FERNIE; STITT, 2012). Metabolomics is thus an important tool to unveil how plants respond to stress conditions (OBATA; FERNIE, 2012), given that it may unveil initial responses not observed in other levels. Furthermore, metabolomics is highly effective in unveiling silent phenotypes (WECKWERTH et al., 2004; DALOSO et al., 2015; FONSECA-PEREIRA et al., 2020). In this context, no morphological symptoms were observed in different incompatible CPSMV-cowpea systems (VARELA et al., 2017, 2019), including the CPSMV-Macaibo pathosystem investigated here (Figure 1A). However, despite the lack of visual symptoms, our metabolomics analyses reveal that CPSMV inoculation leads to several metabolic changes, especially at the primary metabolism level and in the directly infected leaves. This highlights the importance and the power of metabolomics to unveil the mechanisms behind plant stress tolerance.

2.5.2 CPSMV infection alters especially the primary rather than the secondary metabolism

Plant metabolism is separated in primary and secondary metabolisms. Whilst the first is related to essential metabolic pathways such as those associated with carbon and nitrogen metabolisms, the former is especially activated under (a)biotic stress conditions (SAITO et al., 2013). The balance between the accumulation of primary and secondary metabolites is thus a great indicator of the growth-stress tolerance trade-off (BECHTOLD; FIELD, 2018; CÂNDIDO-SOBRINHO et al., 2022; FUSARI et al., 2017; LIU et al., 2019). However, a growing body of evidence highlights that the primary metabolism is of paramount importance for plant stress acclimation (BALFAGÓN et al., 2022; BARROS et al., 2017;

CARDOSO; FREIRE; DALOSO, 2022; FÀBREGAS et al., 2018; FÀBREGAS; FERNIE, 2019; PIRES et al., 2016; ZANDALINAS et al., 2022). Here, we used two different metabolomics platforms namely GC/MS and LC/MS that mostly detect primary and secondary metabolites, respectively (LISEC et al., 2006; PEREZ DE SOUZA et al., 2021). Our results highlight that the major changes induced by CPSMV infection was observed at the primary rather than the secondary metabolism. This is evidenced by the low number of features and metabolites derived from the LC/MS platform that have significant alteration in response to CPSMV (Tables S2-S3), in contrast to the more drastic changes observed in primary metabolites identified by GC/MS (Table 1).

The higher stability of the secondary metabolism might be related to the incompatible nature of the CPSMV-Macaibo pathosystem. This interaction is characterized by a rapid elimination of the CPSMV, as evidenced by the lack of symptoms 15 days after virus inoculation and the reduction in the level of the expression of the CPSMV coat protein over time (Figures 1A-B). In close agreement, no expression of the CPSMV coat protein was detected in leaves of a CPSMV resistant cowpea genotype 6 days after CPSMV infection (VARELA et al., 2019). It seems likely that changes in the level of proteins (VARELA et al., 2017) and primary metabolites are sufficient to struggle CPSMV infection, safeguarding the secondary metabolism. Indeed, evidence suggests that stress-induced changes in the level of primary metabolites are faster than that observed in secondary metabolites (ANGELOVICI; KLIEBENSTEIN, 2022; BRAND; TISSIER, 2022), whose biosynthesis and accumulation is mostly transcriptionally regulated (YANG et al., 2012). However, it remains unclear whether the level of secondary metabolites is constitutively high in Macaibo leaves, which could contribute to CPSMV resistance without the need to further increase the amount of these compounds after CPSMV infection. Further LC/MS analysis comparing susceptible and resistant cowpea genotypes to CPSMV may unveil which metabolism-mediated mechanisms aid cowpea plants to resist CPSMV infection. This information is also important to understand the growth-defence trade-off, given that the productivity of CPSMV resistant cowpea cultivars is lower, compared to the sensitive ones (PAIVA et al., 2014).

2.5.3 On the stability of the primary metabolic network under CPSMV infection

Plant metabolism operates as a highly integrated network (SWEETLOVE; FERNIE, 2005). It is thus crucial to analyse the metabolic changes at network level. Systems biology analysis offers the possibility to unveil emergent properties that modulate plant stress

responses, which cannot be identified using reductionist approaches (AULER et al., 2022; GALVIZ; SOUZA; LÜTTGE, 2022; NETO et al., 2021; SOUZA et al., 2016). Indeed, our network analysis unveil that, although substantial changes have been observed in the accumulation of primary metabolites, especially in local leaves, the values of both density and heterogeneity of the local leaves metabolic network oscillated throughout the time of CPSMV infection, with no pattern of increase or decrease over time, when compared to the mock-treated local leaves (Figure 10). Although local leaves have lower density and higher heterogeneity than mock-treated local leaves in the first day of CPMSV infection (i.e. 2, 4 and 8 HAI) (Figures 10A,C), which is associated to a clear reduction in the number of hubs (Figure 10E), the values of density and heterogeneity were very similar between mock and CPSMV treated local leaves at 24 and 72 HAI (Figures 10A,C). These results indicate that the dynamic of the topology and the density of the metabolic networks of local leaves were not substantially altered by CPSMV infection. Similarly, a recent multi-species/stress condition meta-analysis indicate that the changes in topology and density of metabolic networks are species/stress level specific (CARDOSO; FREIRE; DALOSO, 2022). Cardoso and collaborators demonstrated that there is no pattern of changes in network topology and density in response to stress (CARDOSO; FREIRE; DALOSO, 2022). Thus, although the changes in the level of primary metabolites seems to be a common response to (a)biotic stress conditions (FÀBREGAS; FERNIE, 2019; FONSECA-PEREIRA et al., 2019; OBATA; FERNIE, 2012; PIRES et al., 2016; ZANDALINAS et al., 2022), as we observed here, the metabolic network of cowpea leaves directly infected with CPSMV remained relatively stable throughout the time of CPSMV infection, which might be related to the incompatible CPSMV-Macaibo interaction.

High network stability has been associated to a better capacity of the genotype to cope with stress conditions (SOUZA; LÜTTGE, 2015). This idea relies in the fact that stable networks would avoid the propagation of the noise derived from the stress condition throughout the network, maintaining the stability (homeostasis) of the system (AMZALLAG, 2001; SOUZA; PINCUS; MONTEIRO, 2005; SOUZA; RIBEIRO; PINCUS, 2004). The stability of the system is thus closely associated to its degree of phenotypic plasticity, i.e. to the extent by which the system is able to acclimate to variations in the external environment without the need of genotypic changes (DALOSO, 2014; FREEMAN et al., 2003; LÜTTGE, 2021; SOUZA; LÜTTGE, 2015; VALLADARES et al., 2002). Here, the stability of metabolic network parameters over time of CPSMV infection, considered as the lack of a general pattern of increase or decrease after CPMSV infection, can be interpreted as part of

the stress acclimation mechanism of a CPSMV resistant genotype. It is noteworthy that the stability of a certain level of the system can be obtained by tremendous changes in another level and/or emerge from the interaction between the plasticity and complexity of the different modules that compose the system (SOUZA; LÜTTGE, 2015). Thus, changes in the level of certain primary metabolites were likely sufficient to maintain both the stability of the metabolic network and the homeostasis of the system under CPSMV infection.

2.5.4 Looking to the subnetworks: metabolic pathways associated to CPSMV resistance

Scale-free networks, such as plant metabolic networks, are characterized by the presence of few nodes with high number of connections (links), called hubs (BARABÁSI, 2009). Although the controllability of the network toward a desired outcome apparently resides in nodes with low connection, i.e. not in the hubs of the network (LIU; SLOTINE; BARABÁSI, 2011), the hubs are important for the stability of the network topology and have high probability to be essential to the organism (HELSEN et al., 2019; JEONG et al., 2001; MOHANTY et al., 2016). In plants, amino acids associated to stress acclimation such as Asn, Val, Leu, Ile, Pro, Trp and Gly have been identified as hubs in metabolic networks from numerous species under different stress conditions (CARDOSO; FREIRE; DALOSO, 2022). Here, we investigated which topological change occurs in the metabolic networks and which nodes (metabolites) appear as hubs during CPSMV infection to obtain better insights on how cowpea overcome CPSMV infection. We further integrated this information with the VIP score list obtained by the PLS-DA modelling, which has been shown to be effective in unveiling metabolic pathways that are responsive to different stress conditions (CARDOSO; FREIRE; DALOSO, 2022). When the data was analysed combining all time points (from 2 to 72 HAI), both local and systemic networks showed higher density and lower heterogeneity, when compared to mock-treated leaves (Figure 8). Interestingly, Val and Ile were significantly and positively correlated in mock-treated local leaves (Figure 9A). This strong interaction was maintained after CPSMV infection with addition of Leu and homoserine, creating a module (subnetwork) highly interconnected to each other but not connected to the rest of the metabolic network (Figure 9A). Furthermore, these metabolites were present in diverse VIP score lists of the PLS-DA models (Table S1), which is related to their higher level in CPSMV-inoculated leaves, as compared to mock treated mock leaves, especially at 48 HAI (Figure 5). These results indicate that they are great markers for the discrimination of mock and CPSMV-treated leaves. These results thus indicates that the metabolic pathways

associated to branched chain amino acids (BCAAs) such as Leu, Ile, Val and homoserine are highly responsive to CPSMV infection and might be related to the Macaibo CPSMV resistance.

Homoserine is synthesized from Asp and is a metabolic intermediate of Thr biosynthesis (AUBERT et al., 1998). Thr can be also used to the synthesis of Val, which justify the high correlation among them. Leu, Ile and Val are BCAAs playing an important role defending plants against (a)biotic stress conditions (BATISTA-SILVA et al., 2019; HILDEBRANDT et al., 2015). They can be synthesized from pyruvate and from protein degradation under stress condition, which aid plants to cope with stress conditions (ARAÚJO et al., 2011). The carbons released from these metabolic pathways, and others associated to autophagy processes, are used to feed the TCA cycle and the oxidative phosphorylation system (OXPHOS) via the electron-transfer flavoprotein/electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF/ETFQO) pathway, an alternative respiratory metabolic pathway (AVIN-WITTENBERG, 2019; BARROS et al., 2020; MICHAELI et al., 2016). The activation of the ETF/ETFQO pathway has been shown as an important mechanism for plant acclimation to water deficit and extended dark conditions (ARAÚJO et al., 2010; BARROS et al., 2017, 2021; PIRES et al., 2016) as well as for normal seed development and germination (DA FONSECA-PEREIRA et al., 2022). Beyond being found as a prominent module in local CPSMV-infected leaves, the dynamic of the accumulation of Val, Ile and Leu was also altered by CPSMV. Furthermore, the level of these BCAAs and TCA-cycle metabolites and related amino acids such as citrate, fumarate, pyruvate, Ala, Asn, Glu, Gln and Pro were also altered after CPSMV infection in local leaves, especially at 48 HAI (Figure 5). These results, coupled to the role of TCA cycle metabolites, BCAAs and other amino acids for plant stress acclimation (HILDEBRANDT et al., 2015), suggest that the interplay between the TCA cycle and the amino acid metabolism greatly contribute to CPSMV tolerance in Macaibo plants. Further studies aiming to manipulate such metabolic pathways in CPSMV sensitive cowpea genotypes may unveil their biotechnological potential in obtaining CPSMV tolerant genotypes.

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2.7 Figures Chapter I

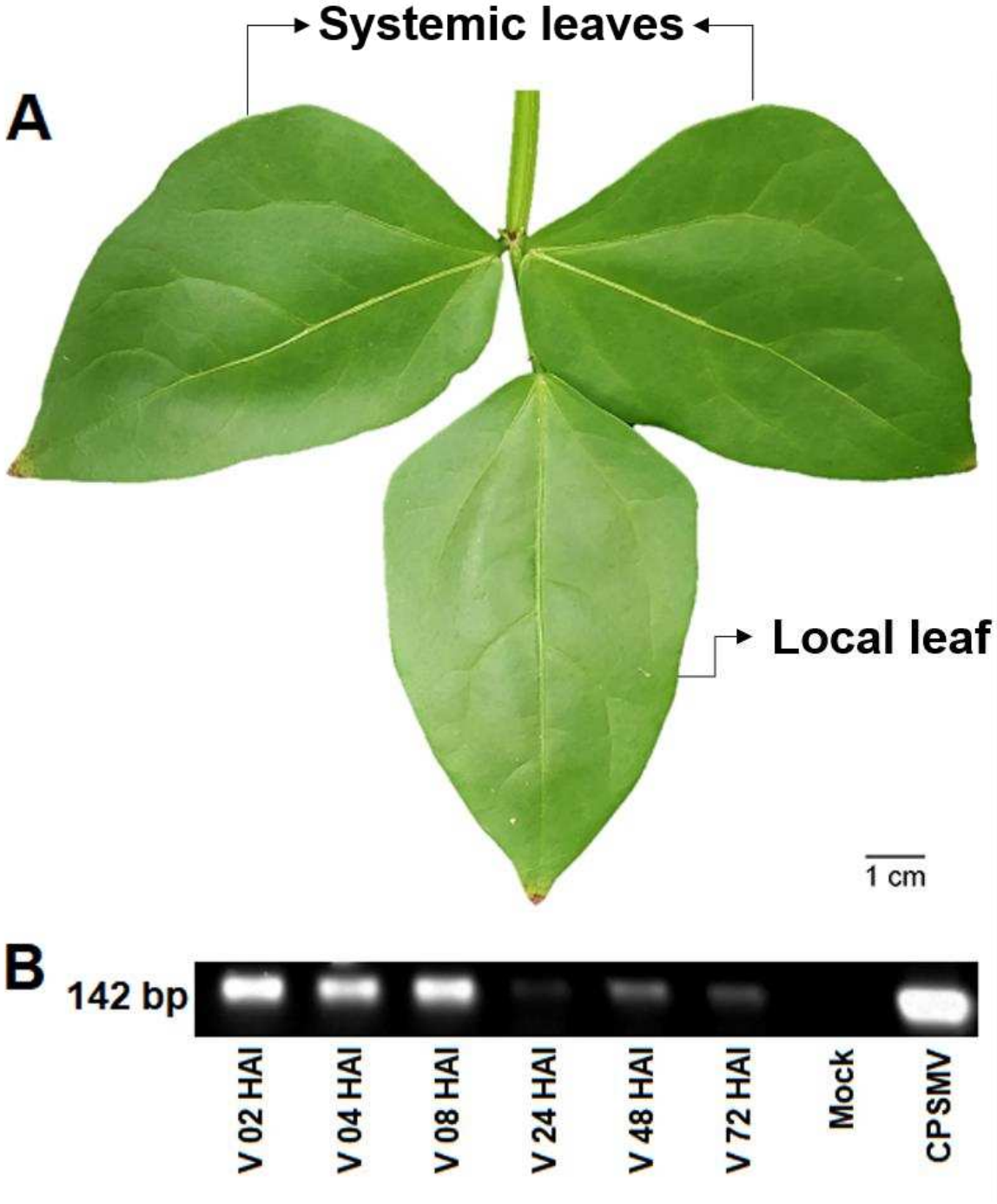

Figure 1 Representative leaf infected with cowpea severe mosaic virus (CPSMV) and the detection of the CPSMV in leaf tissues of the Macaibo genotype. A) Representative image of a cowpea first true leaf 15 days after CPSMV inoculation. B) Detection of the coat protein of CPSMV by PCR analysis using CPSMV inoculated leaves after 2, 4, 8, 24, 48 and 72 hours after inoculation (HAI). Mock and CPSMV lanes represent negative and positive controls. The band found in the CPSMV spot corresponds to a leaf material from a CPSMV-susceptible genotype.

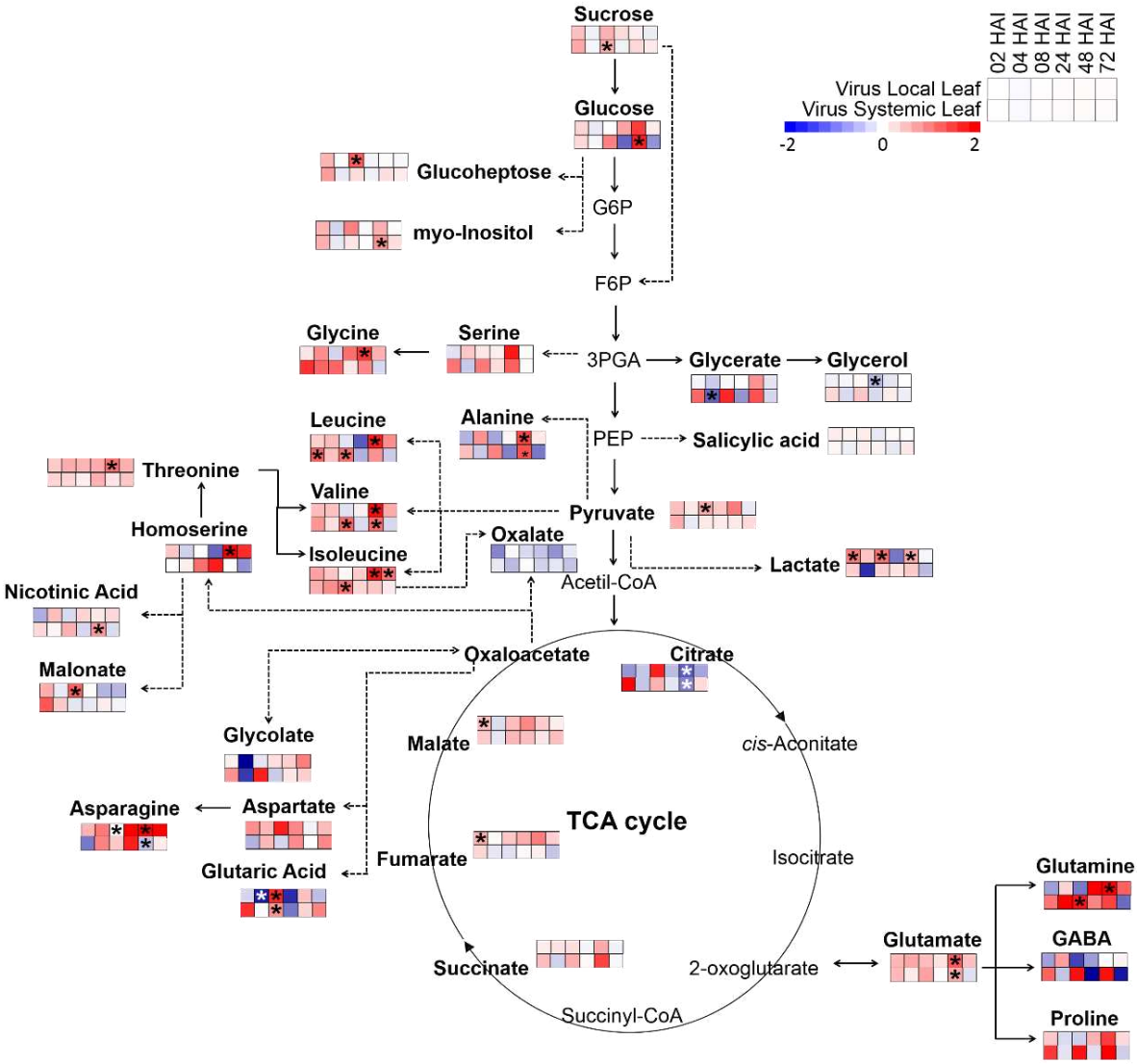

Figure 2 Changes in the level of primary metabolites induced by cowpea severe mosaic virus (CPSMV) infection. Local and systemic leaves were subjected to mock or CPSMV treatments and harvest after 02, 04, 08, 24, 48 and 72 hours. The level of each metabolite is represented by the colour scale of the legend. Red and blue squares represent metabolites with increased and decreased level in CPSMV-infected local (upper squares) and systemic (lower squares) leaves, as compared to their mock control, respectively. Dashed lines indicate non-sequential metabolic reactions. Abbreviation: HAI, hours after inoculation.

Table 1. List of metabolites identified by gas chromatography mass spectrometry (GC/MS) analysis in local and systemic leaves infected with Mock or CPSMV (cowpea severe mosaic virus). The pattern of accumulation of the metabolites over time was analysed by analysis of variance (ANOVA) ($P < 0.05$) using the entire data set (all time points) of local and systemic leaves infected with Mock or CPSMV. Metabolites with no changes over time are identified with ns (non-significant). These results are displayed in the Time columns. Metabolites with differential accumulation between Mock and CPSMV-infected leaves were compared by Student´s *t* test ($P < 0.05$) in each time point (02, 04, 08, 24, 48 and 72 hours after infection). The columns Mock *vs* CPSMV display metabolites in which the level was significantly ($P < 0.05$) or non-significantly (ns) altered in CPSMV compared to Mock in at least one time point.

	Time		**Mock *vs* CPSMV**	
Metabolites	**Local**	**Systemic**	**Local**	**Systemic**
Alanine	$P < 0.05$	ns	$P < 0.05$	ns
Asparagine	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Aspartate	Ns	ns	$P < 0.05$	ns
Butyrate	ns	$P < 0.05$	ns	ns
Citrate	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Fumarate	$P < 0.05$	$P < 0.05$	$P < 0.05$	ns
Glucoheptose	ns	ns	$P < 0.05$	ns
Glucose	$P < 0.05$	$P < 0.05$	ns	$P < 0.05$
Glutamate	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Glutamine	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Glutarate	$P < 0.05$	ns	$P < 0.05$	$P < 0.05$
Glycerate	ns	ns	ns	$P < 0.05$
Glycerol	$P < 0.05$	ns	$P < 0.05$	ns
Glycine	$P < 0.05$	$P < 0.05$	$P < 0.05$	ns
Glycolate	ns	ns	ns	ns
Homoserine	$P < 0.05$	$P < 0.05$	$P < 0.05$	ns
myo-inositol	ns	ns	ns	$P < 0.05$
Isoleucine	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Lactate	$P < 0.05$	ns	$P < 0.05$	ns
Leucine	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Malate	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Malonate	ns	ns	$P < 0.05$	
Nicotinic_acid	ns	$P < 0.05$	ns	$P < 0.05$
Oxalate	$P < 0.05$	$P < 0.05$	ns	ns
Proline	$P < 0.05$	$P < 0.05$	ns	ns
Pyruvate	$P < 0.05$	ns	$P < 0.05$	ns
Salicylic_acid	ns	ns	ns	ns
Serine	$P < 0.05$	$P < 0.05$	ns	ns
Succinate	ns	ns	ns	ns
Sucrose	$P < 0.05$	ns	ns	$P < 0.05$
Threonine	$P < 0.05$	$P < 0.05$	$P < 0.05$	ns
Valine	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

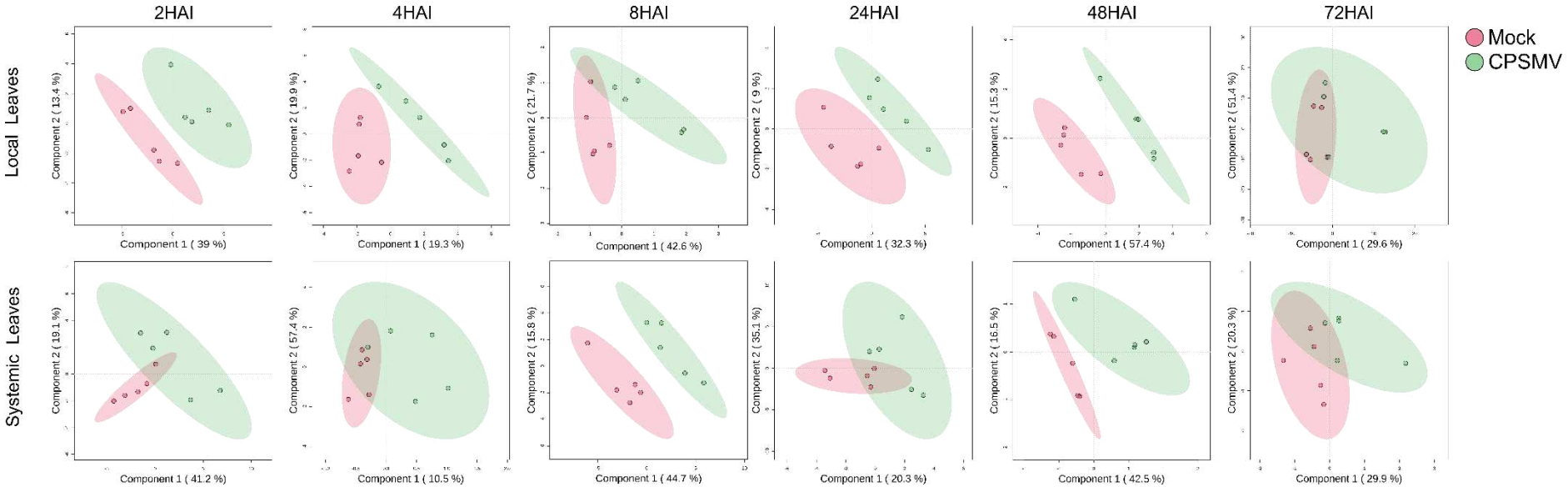

Figure 3 Partial least-squares discriminant analysis (PLS-DA) using raw data of the gas chromatography mass spectrometry (GC/MS)-based metabolite profiling analysis. Raw data refer to the level of the metabolites normalized by the ribitol and the fresh weight used in the extraction. Local (upper panel) and systemic (lower panel) leaves were subjected to mock (in red) or cowpea severe mosaic virus (in green) treatments and harvest after 02, 04, 08, 24, 48 and 72 hours. The percentage variation explained by the PC1 and PC2 are represented in each axis. PLS-DA was carried out using the Metaboanalyst platform (n = 5). Abbreviation: HAI, hours after inoculation

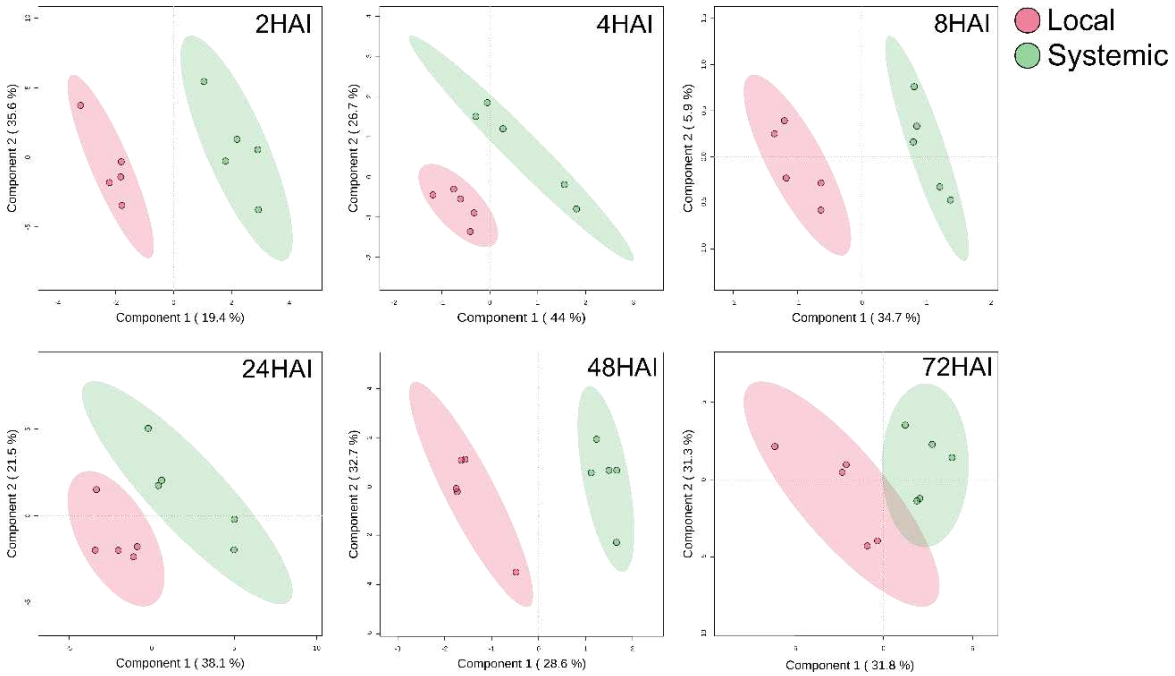

Figure 4 Partial least-squares discriminant analysis (PLS-DA) using gas chromatography mass spectrometry (GC/MS)-based metabolite profiling data. Local (in red) and systemic (in green) leaves were subjected to mock or cowpea severe mosaic virus (CPSMV) treatments and harvest after 02, 04, 08, 24, 48 and 72 hours. This PLS-DA was carried out using data from local and systemic leaves infected with CPSMV normalized by their respective mock control. The percentage variation explained by the PC1 and PC2 are represented in each axis. PLS-DA was carried out using the Metaboanalyst platform (n = 5). Abbreviation: HAI, hours after inoculation

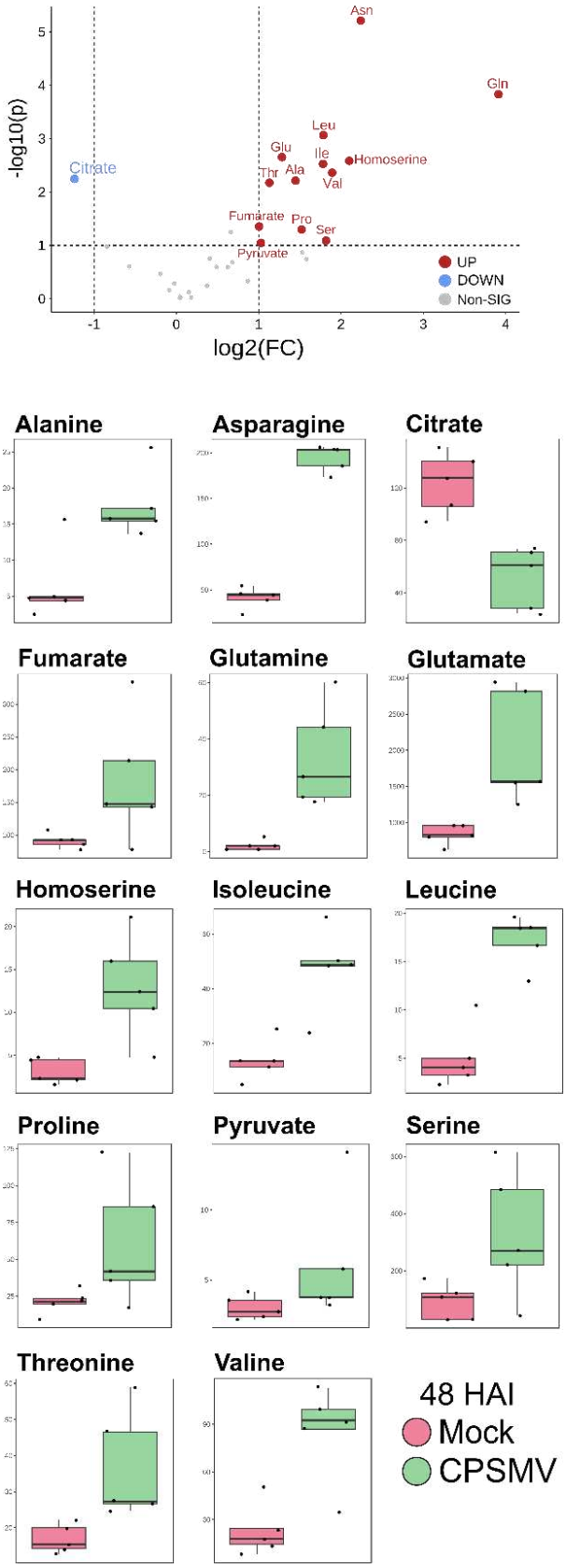

Figure 5 Volcano and box plots of selected metabolites identified by gas chromatography mass spectrometry (GC/MS) analysis in local leaves inoculated with Mock (control group) or CPSMV (cowpea severe mosaic virus) for 48 h. These metabolites showed a differential accumulation between mock and CPSMV treatments by Student´s t test ($P < 0.05$). This analysis was carried out using the Metaboanalyst platform.

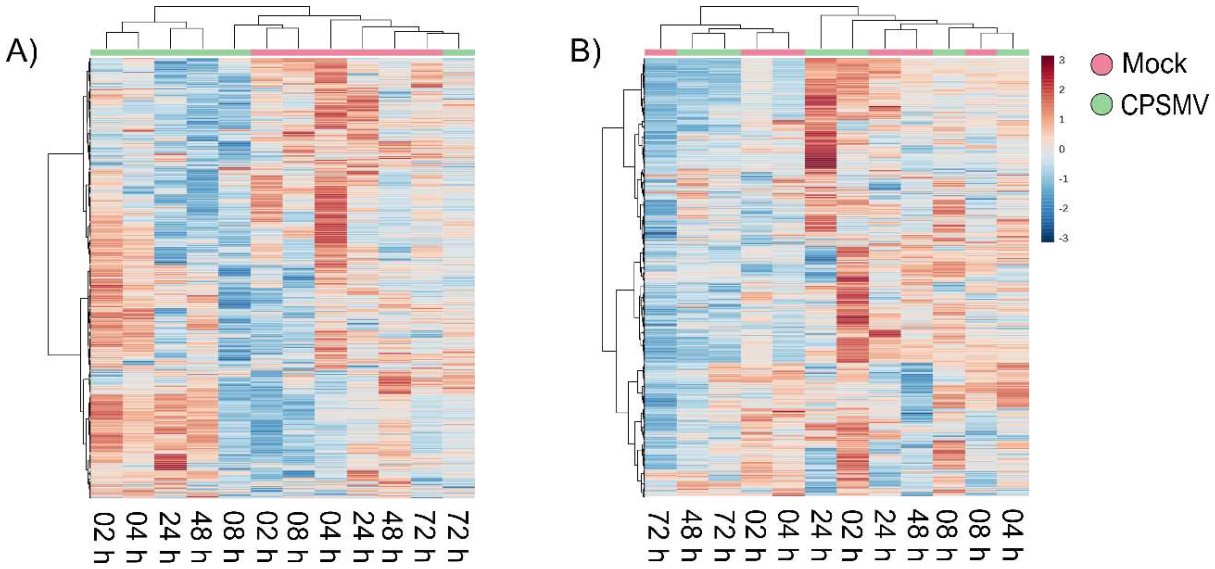

Figure 6 Heatmap representation of the liquid chromatography mass spectrometry (LC/MS)-based metabolic fingerprinting analysis. Local (a) and systemic (b) leaves were subjected to mock (Mock) or cowpea severe mosaic virus (CPSMV) treatments and harvest after 02, 04, 08, 24, 48 and 72 h. The heatmap was created using raw values, which refer to the level of the features normalized by the internal control and the fresh weight used in the extraction. These analyses were carried out using the Metaboanalyst platform.

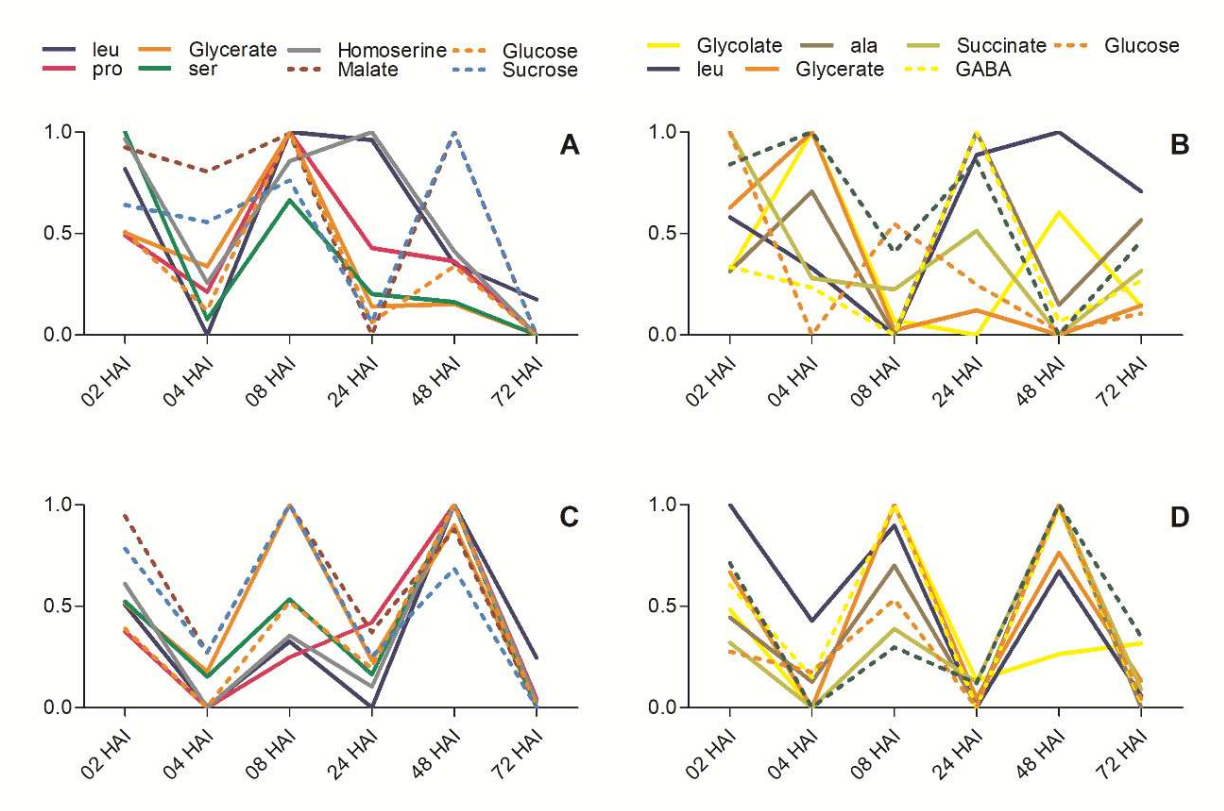

Figure 7. DTW of virus inoculated local leaves. Snippet of the dynamic time wrapping analysis (DTW; Supplementary 7) displaying only the virus inoculates local leaves that exhibited a different pattern of its mocks. DTW analysis were performed using Time Series Clustering Utilities (TSclust) on R software using the metabolite amounts converted to a function of maximum (= 1) and minimum (= 0) on Microsoft Excel software. Metabolites were divided in a total of 20 clusters, accordingly to its accumulation pattern over time (02, 04, 08, 24, 48, 72 HAI). Each data point represents the mean of five independent biological replicates. Original clusters: (A) 03, (B) 06, (C) 10, (D) 11, (E) 12, (F) 13, (G) 14, (H) 15. Abbreviation: HAI, hours after inoculation

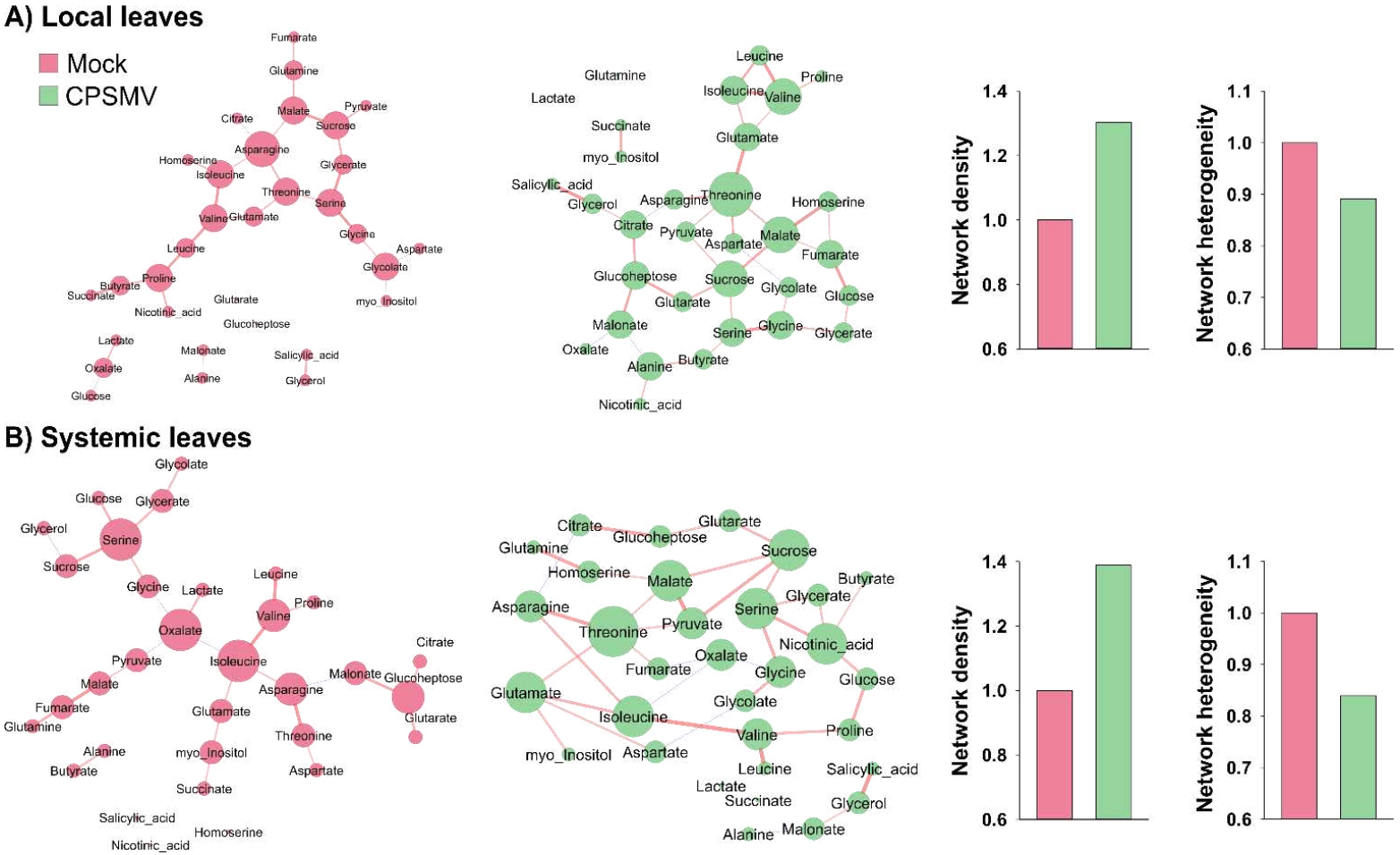

Figure 8 Correlation-based metabolic networks of local (upper panel) and systemic (lower panel) leaves subjected to mock (blue nodes) or cowpea severe mosaic virus (CPSMV) (red nodes) treatments. The metabolic networks were created combining gas chromatography mass spectrometry (GC/MS)-based metabolite profiling data from all time points (from 02 to 72 hours after inoculation). Nodes and links represent metabolites and the correlation among them, respectively. Bigger nodes and thicker lines imply more connections and stronger debiased sparse partial correlation (DSPC) coefficient (*r*), respectively. Red and blue lines represent positive and negative *r*. The networks were build using MetScape on Cytoscape software considering -0.5 > *r* > 0.5. Both density and heterogeneity of the networks were obtained by using NetworkAnalyzer on Cytoscape.

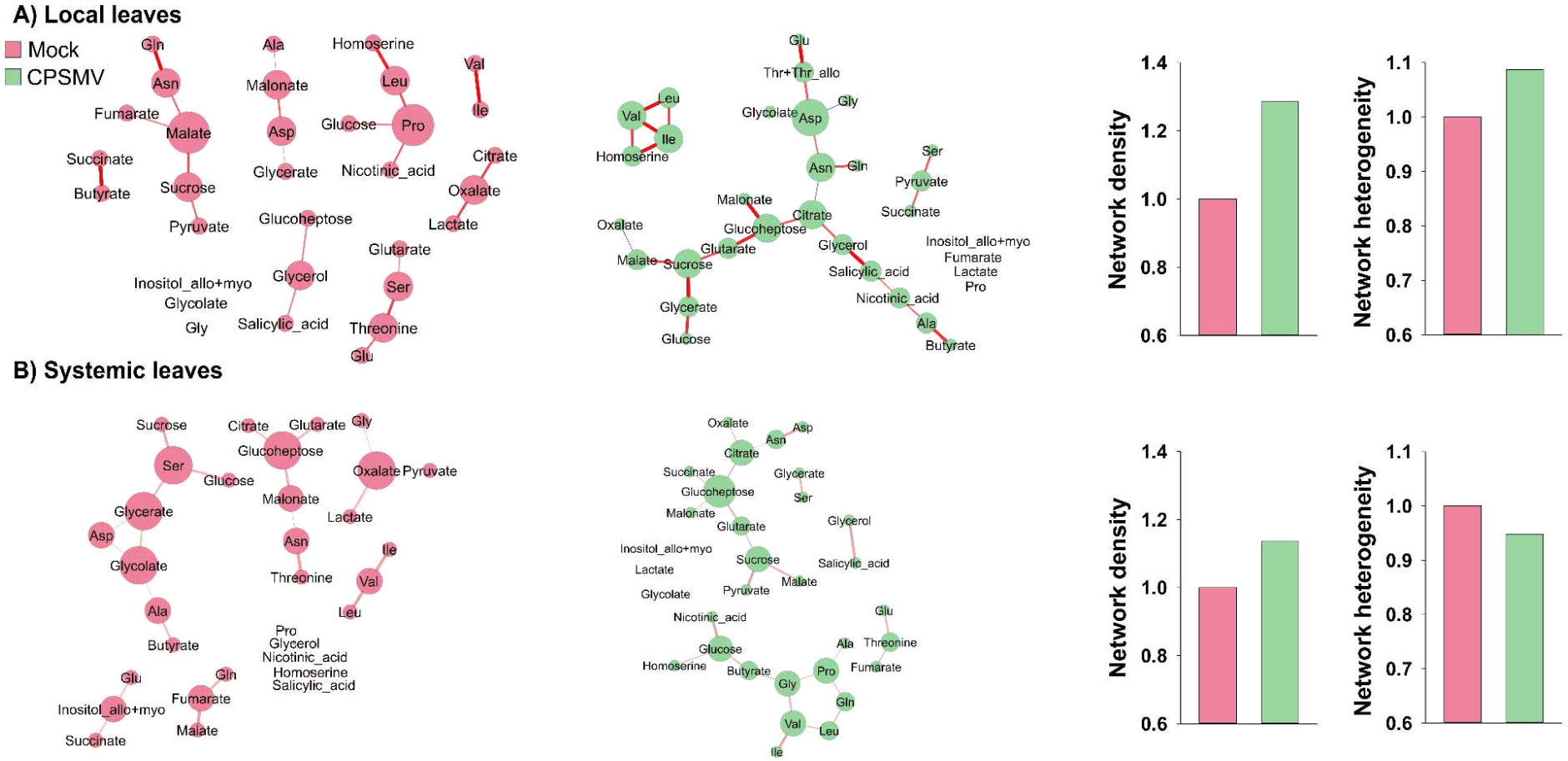

Figure 9 Correlation-based metabolic networks of local (upper panel) and systemic (lower panel) leaves subjected to mock (blue nodes) or cowpea severe mosaic virus (CPSMV) (red nodes) treatments. The metabolic networks were created combining gas chromatography mass spectrometry (GC/MS)-based metabolite profiling data from all time points (from 02 to 72 hours after inoculation). Nodes and links represent metabolites and the correlation among them, respectively. Bigger nodes and thicker lines imply more connections and stronger debiased sparse partial correlation (DSPC) coefficient (r), respectively. Red and blue lines represent positive and negative r. The networks were build using MetScape on Cytoscape software considering only the significant correlations ($P < 0.05$). Both density and heterogeneity of the networks were obtained by using NetworkAnalyzer on Cytoscape.

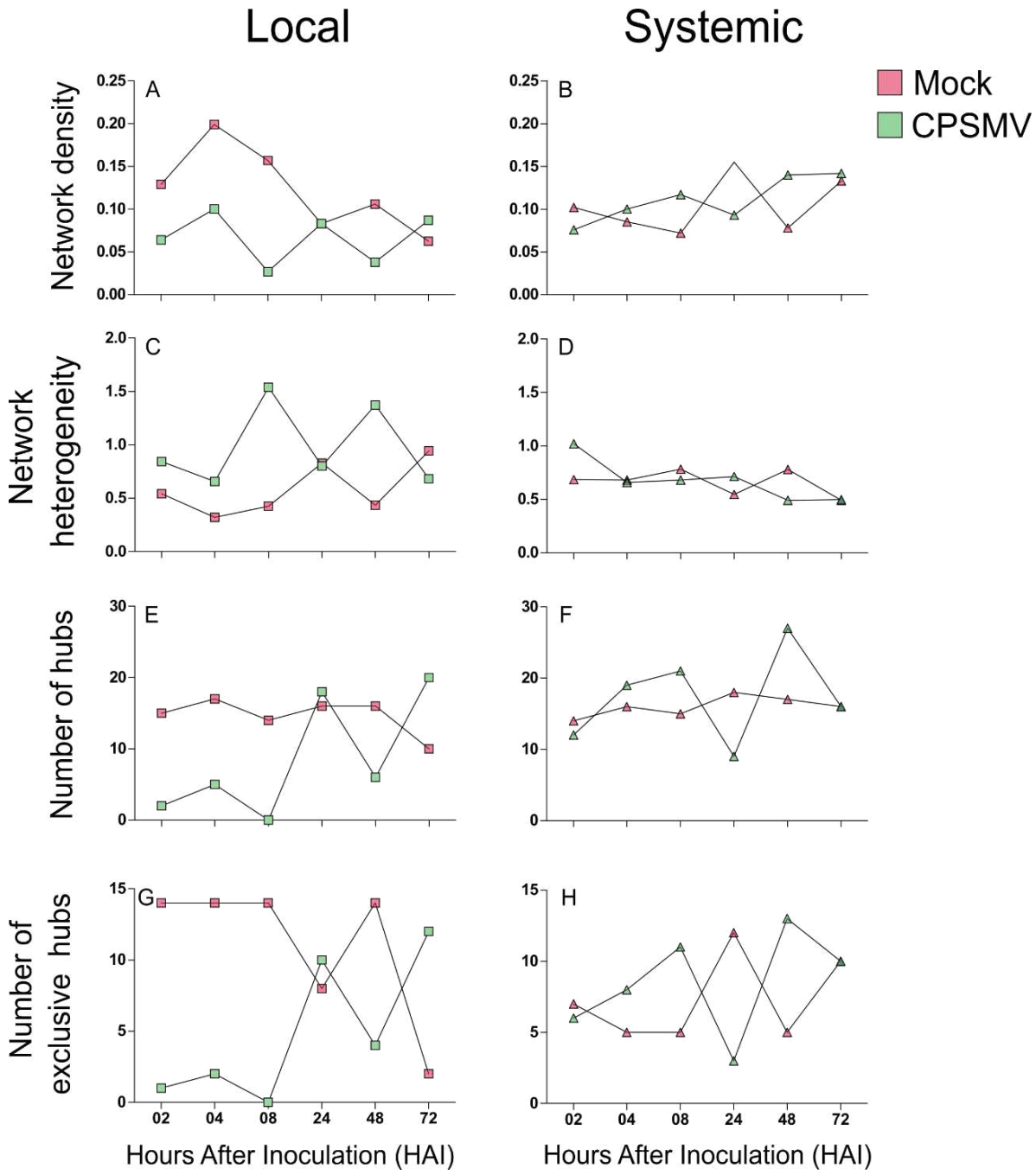

Figure 10 Parameters obtained from correlation-based metabolic networks of mock inoculated local (ML) and systemic (MS) leaves, and virus inoculated local (VL) systemic (VS) leaves over time (02, 04, 08, 24, 48, 72 HAI). A-B) Network density. C-D) Network heterogeneity. E-F) Number of hubs of the networks. G-H) Number of exclusive hubs of the networks. The density and heterogeneity of the networks were obtained by using NetworkAnalyzer in the Cytoscape software, while the other parameters were calculated as described earlier (Cardoso et al. 2022).

2.8 Supplemental Figures Chapter I

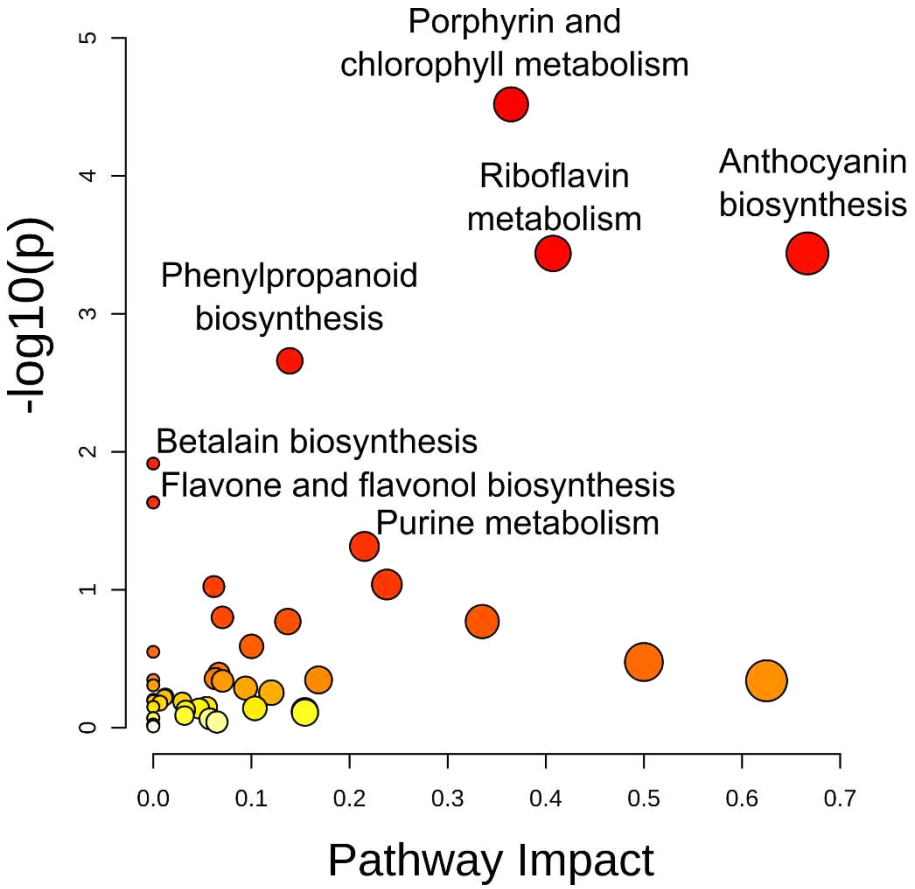

Figure S1 Pathway enrichment analysis. Circles in the figure highlights the different metabolic pathways that the features identified by LC/MS belongs to. The circles are displayed according to the scores from enrichment (y axis) and topology analysis (x axis). The most representative pathways were identified in the graphs, all of them belonging to the secondary metabolism, in agreement with the LC/MS platform used that mostly detect secondary metabolites (PEREZ DE SOUZA et al., 2021). These analyses were carried out using the Metaboanalyst platform.

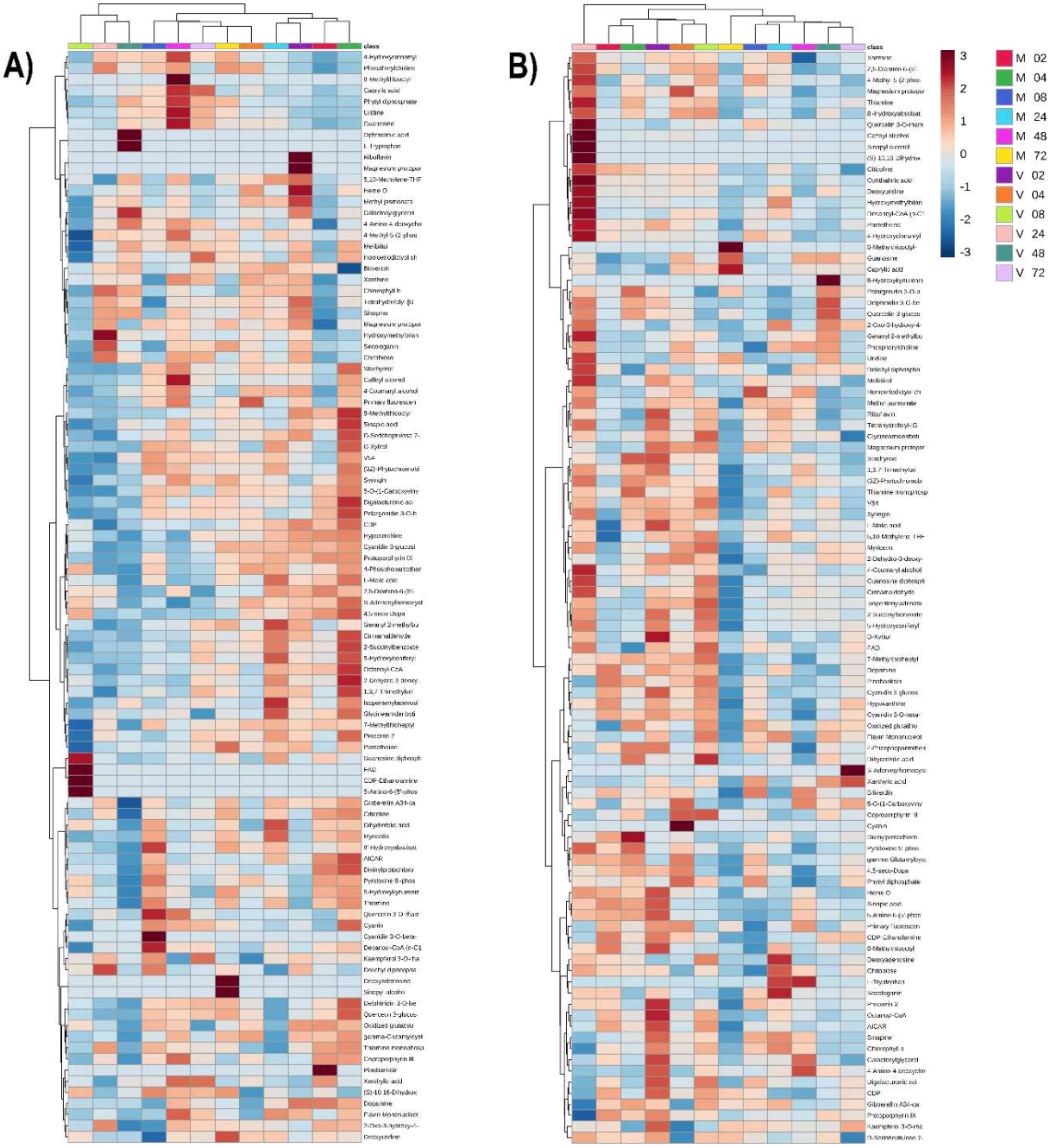

Figure S2 Heatmap representation of the liquid chromatography mass spectrometry (LC/MS)-based metabolite profiling analysis using raw data, which refer to the level of the metabolites normalized by the internal control and the fresh weight used in the extraction. Local (a) and systemic (b) leaves were subjected to mock (M) or cowpea severe mosaic virus (V) treatments and harvest after 02, 04, 08, 24, 48 and 72 hours. The complete list of metabolites identified by LC-MS is found in the table S3. The heatmap was carried out using the Metaboanalyst platform (n = 5).

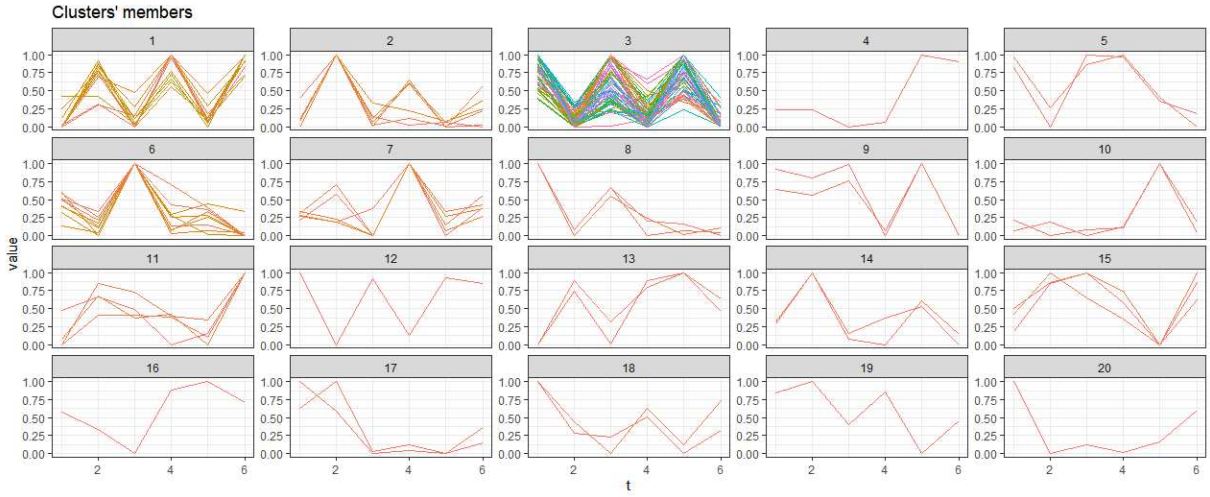

Figure S3 Dynamic time wrapping (DTW) analysis of the gas chromatography mass spectrometry (GC/MS)-based metabolite profiling analysis. Local and systemic leaves were subjected to mock or cowpea severe mosaic virus treatments and harvest after 02, 04, 08, 24, 48 and 72 hours. DTW was performed using Time Series Clustering Utilities (TSclust) on R software using the metabolite amounts converted to a function of maximum (= 1) and minimum (= 0). Metabolites were divided in 20 clusters, accordingly to its accumulation pattern over the six time points (02, 04, 08, 24, 48, and 72 hours after inoculation). The list of metabolites found in each cluster is found in the table S4.

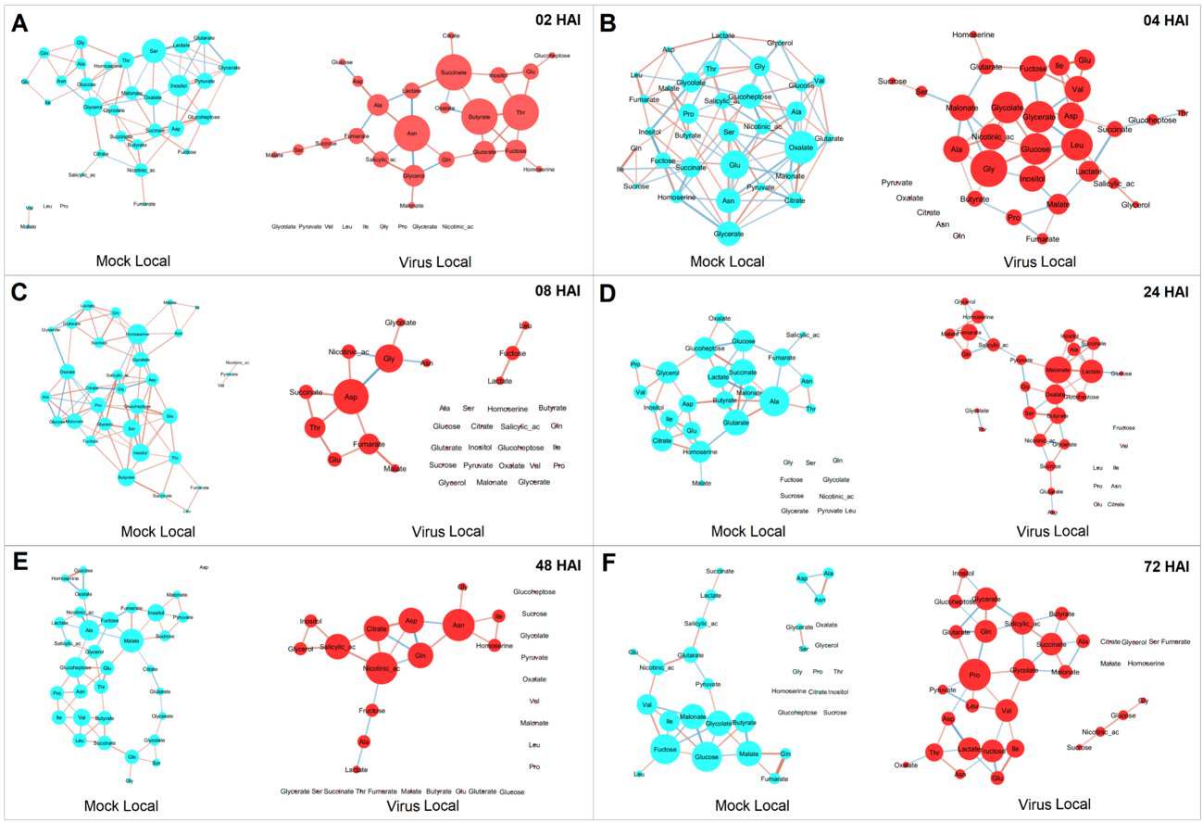

Figure S4 Correlation-based metabolic networks of local leaves. Correlation-based metabolic networks were created using GC/MS-based metabolite profiling data from mock (blue nodes) and CPSMV (red nodes) inoculated local leaves. The networks correspond to the data obtained after 02 (A), 04 (B), 08 (C), 24 (D), 48 (E) and 72 (F) hours after inoculation (HAI). Nodes represent metabolites and the arrows the correlation between them. Bigger nodes indicate more connections (higher degree of connection). Thicker lines indicate stronger correlation, while blue and red lines indicate negative and positive correlations, respectively. The correlations were calculated using the CorrelationCalculator software. The networks were created using MetScape on CYTOSCAPE software considering $-0.5 > r > 0.5$.

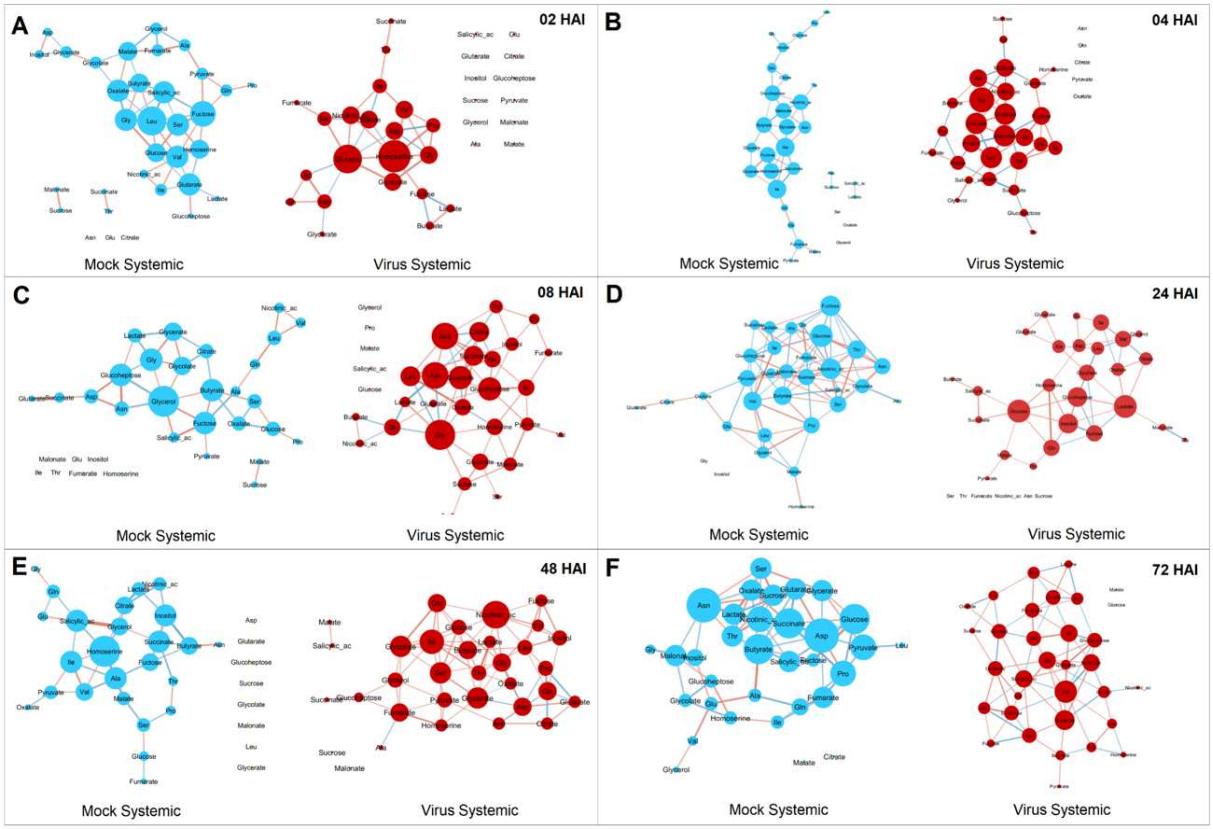

Figure S5 Correlation-based metabolic networks of systemic leaves. Correlation-based metabolic networks were created using GC/MS-based metabolite profiling data from mock (blue nodes) and CPSMV (red nodes) inoculated systemic leaves. The networks correspond to the data obtained after 02 (A), 04 (B), 08 (C), 24 (D), 48 (E) and 72 (F) hours after inoculation (HAI). Nodes represent metabolites and the arrows the correlation between them. Bigger nodes indicate more connections (higher degree of connection). Thicker lines indicate stronger correlation, while blue and red lines indicate negative and positive correlations, respectively. The correlations were calculated using the CorrelationCalculator software. The networks were created using MetScape on CYTOSCAPE software considering $-0.5 > r > 0.5$.

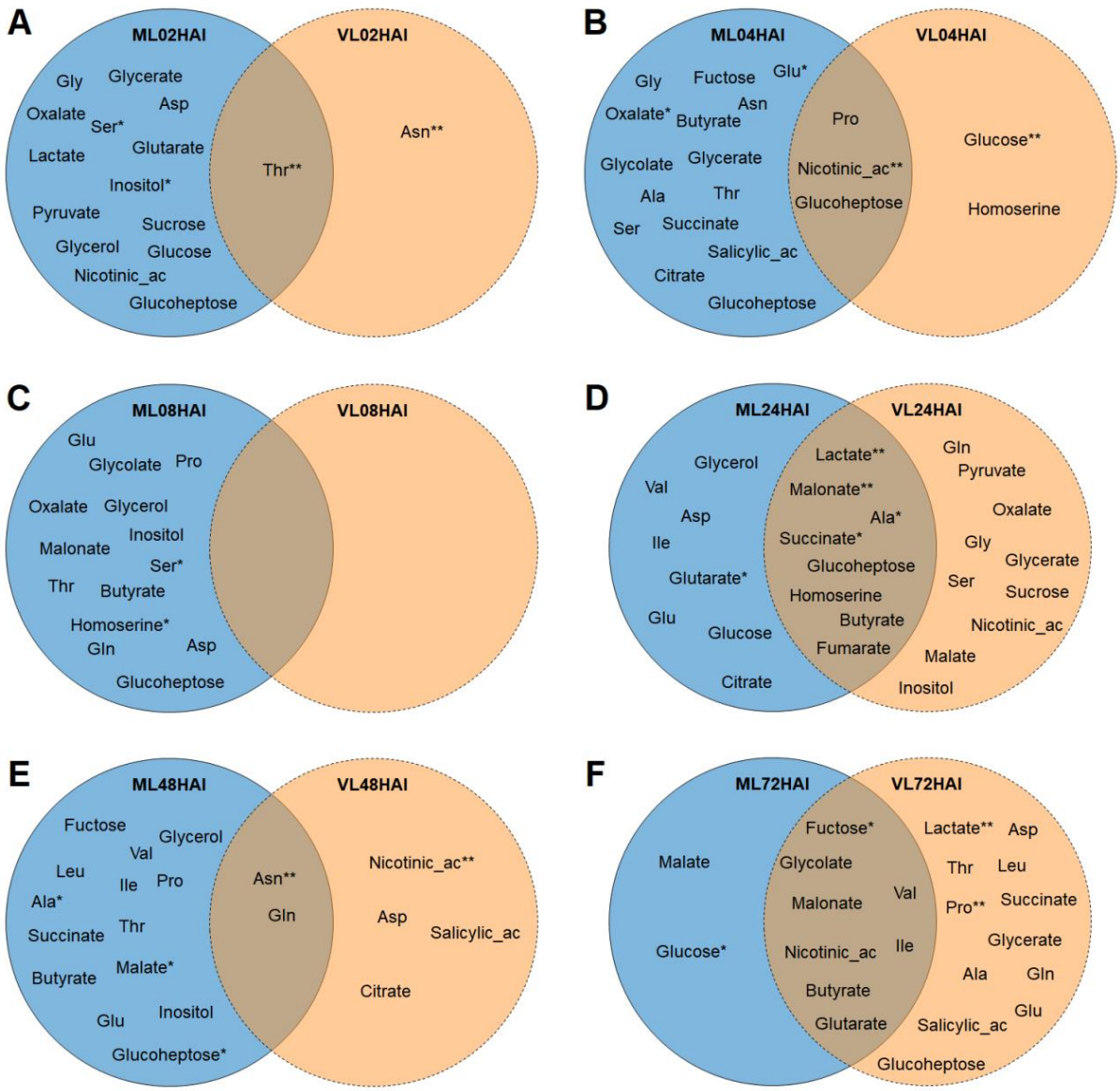

Figure S6 Hubs of local leaf metabolic networks. Venn diagram of the hubs of the metabolic networks of mock (ML) and virus (VL) inoculated local leaves at (A) 02 HAI, (B) 04 HAI, (C) 08 HAI, (D) 24 HAI, (E) 48 HAI, and (F) 72 HAI. One asterisk (*) indicates most important hubs for mock networks, while two asterisks (**) indicates most important hubs for CPSMV networks, and *** indicates important hubs for both mock and CPSMV networks.

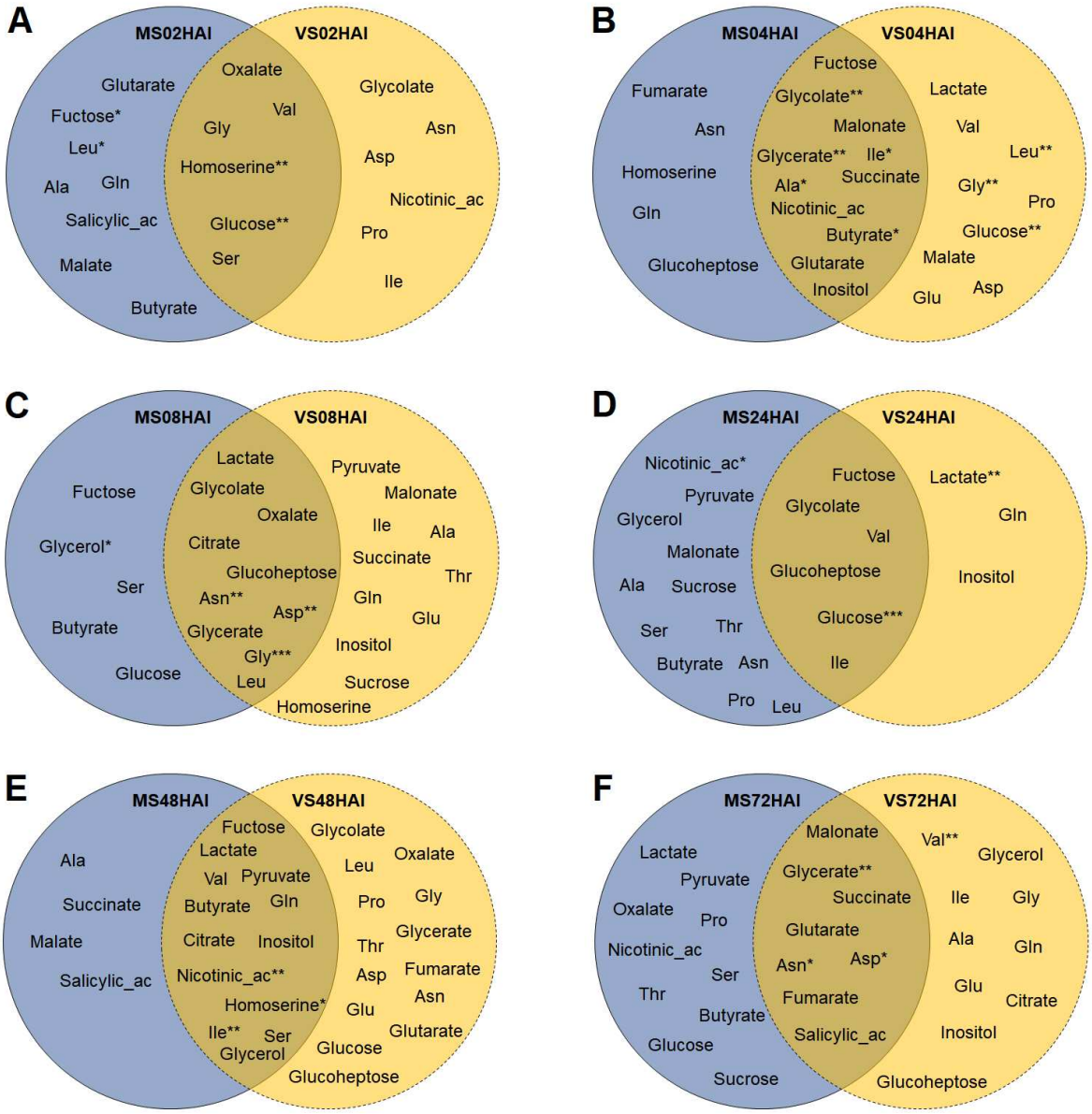

Figure S7 Hubs of systemic leaf metabolic networks. Venn diagram of the hubs of the metabolic networks of mock (ML) and virus (VL) inoculated local leaves at (A) 02 HAI, (B) 04 HAI, (C) 08 HAI, (D) 24 HAI, (E) 48 HAI, and (F) 72 HAI. One asterisk (*) indicates most important hubs for mock networks, while two asterisks (**) indicates most important hubs for CPSMV networks, and *** indicates important hubs for both mock and CPSMV networks.

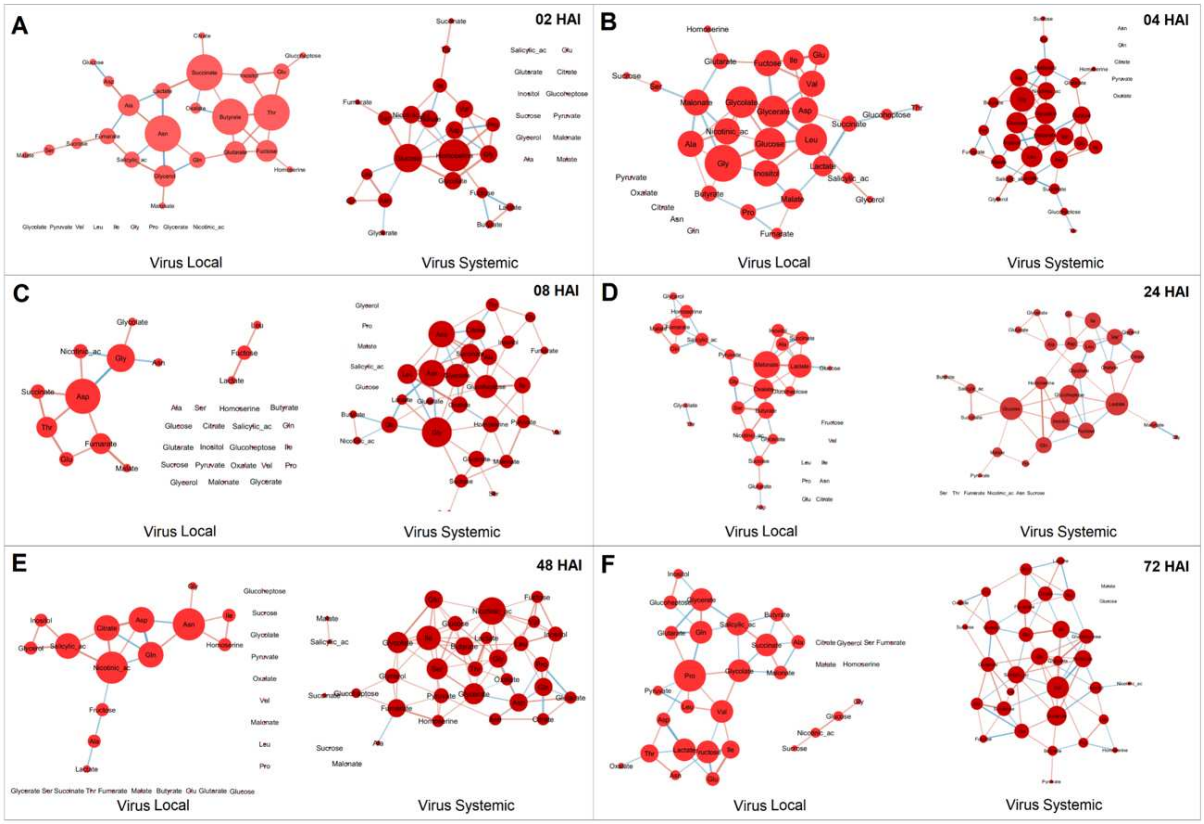

Figure S8 Correlation-based metabolic networks of virus inoculated local and systemic leaves. Correlation-based metabolic networks were created using GC/MS-based metabolite profiling data from CPSMV inoculated local (light red nodes) and systemic (dark red nodes) leaves. The networks correspond to the data obtained after 02 (A), 04 (B), 08 (C), 24 (D), 48 (E) and 72 (F) hours after inoculation (HAI). Nodes represent metabolites and the arrows the correlation between them. Bigger nodes indicate more connections (higher degree of connection). Thicker lines indicate stronger correlation, while blue and red lines indicate negative and positive correlations, respectively. The correlations were calculated using the CorrelationCalculator software. The networks were created using MetScape on CYTOSCAPE software considering $-0.5 > r > 0.5$.

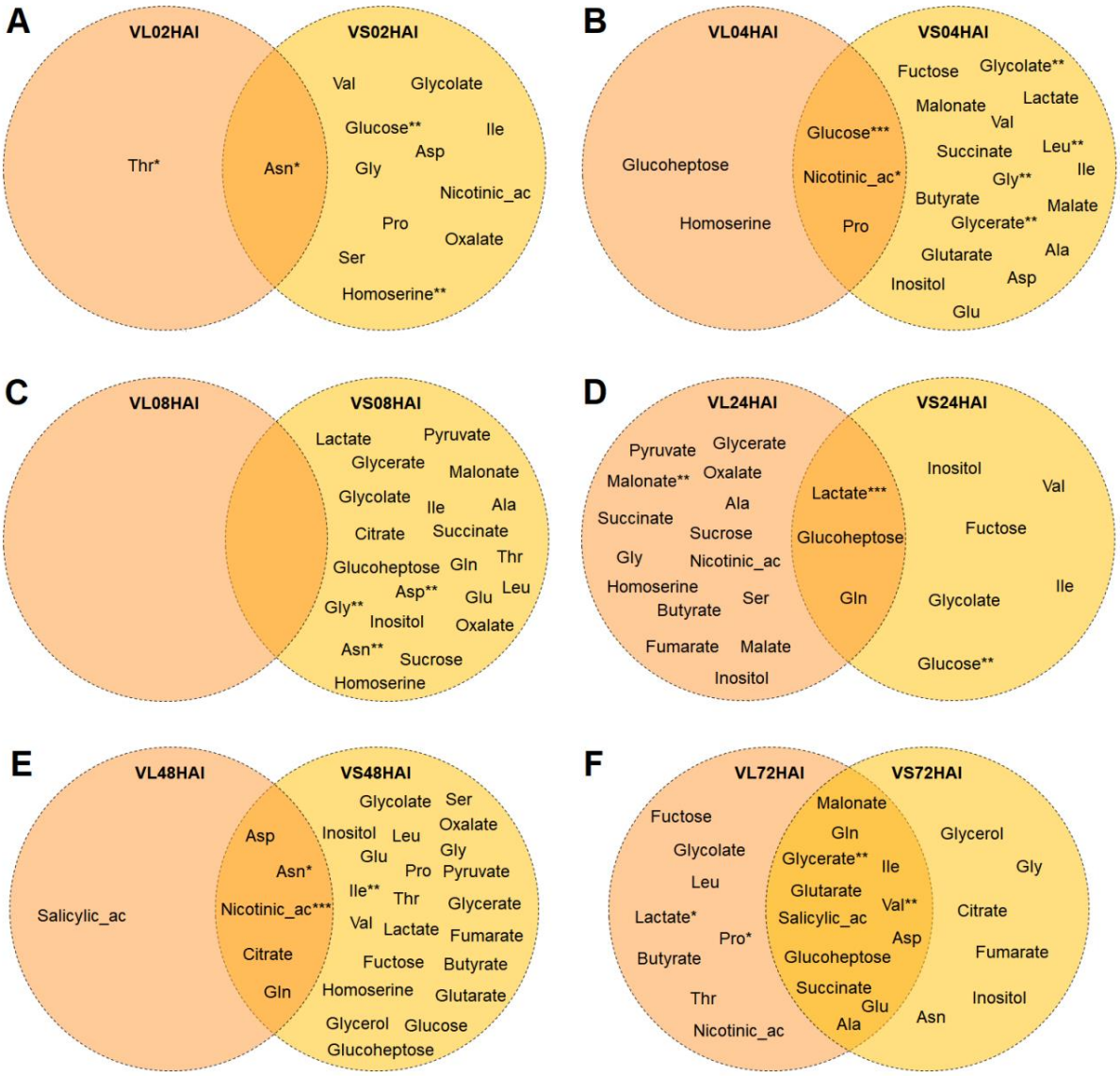

Figure S9 Hubs of CPSMV inoculated metabolic networks. Venn diagram of the hubs of the metabolic networks of virus inoculated local (VL) and systemic (VS) leaves at (A) 02 HAI, (B) 04 HAI, (C) 08 HAI, (D) 24 HAI, (E) 48 HAI, and (F) 72 HAI. One asterisk (*) indicates most important hubs for VL networks, while two asterisks (**) indicates most important hubs for VS networks, and *** indicates important hubs for both VL and VS networks.

Table S1. Metabolites with variable importance in projection (VIP) scores equal or higher than 1.0, obtained from partial least squares-discriminant analysis (PLS-DA) of mock and virus inoculated local and systemic leaves. The metabolites are listed in descendent order of the VIP score.

VIP Score					
Local Leaves					
02 HAI	04 HAI	08 HAI	24 HAI	48 HAI	72 HAI
Lactate	Glutarate	Asp	Glycerol	Asn	Ile
Fumarate	Nicotinic Ac.	Pyruvate	Lactate	Gln	Val
Malate	Thr	Glutarate	Glutarate	Leu	Glu
Glucoheptulose	Glu	Glucoheptulose	Malate	Glu	Citrate
Nicotinic Ac.	Sucrose	Malonate	Gly	Homoserine	Pro
Glu	Succinate	Lactate	Salicylic Ac.	Ile	Leu
Pyruvate	Citrate	Citrate	Nicotinic Ac.	Val	Glutarate
Ile	Ala	Inositol	Asn	Citrate	Nicotinic Ac.
Thr	Lactate	Malate	Fumarate	Ala	Fumarate
Salicylic Ac.	Pyruvate	Ala	Pyruvate	Thr	Malonate
Inositol	Gly	Glu	Citrate	Fumarate	Homoserine
Oxalate		Gln	Oxalate	Pro	
Asn		Thr	Leu	Lactate	
Val					
Systemic Leaves					
02 HAI	04 HAI	08 HAI	24 HAI	48 HAI	72 HAI
Leu	Succinate	Gln	Glucose	Inositol	Glucose
Butyrate	Ile	Leu	Ala	Glu	Butyrate
Val	Glycolate	Ile	Glycolate	Glucose	Salicylic Ac.
Sucrose	Oxalate	Val	Butyrate	Nicotinic Ac.	Nicotinic Ac.
Ile	Pro	Sucrose	Nicotinic Ac.	Ala	Glutarate
Citrate	Leu	Homoserine	Glutarate	Val	Asp
Malate	Citrate	Glutarate	Glycerol	Pro	Ala
Inositol	Val	Butyrate	Asp	Asn	Gln
Gly	Malonate	Glycolate	Homoserine	Butyrate	Inositol
Pyruvate	Glycerate	Glycerate	Leu	Leu	Glu
Glycolate	Pyruvate	Malate		Glycerate	Homoserine
Malonate	Ser	Gly		Ser	Citrate
Ser	Butyrate			Succinate	Lactate
Pro				Ile	Fumarate
Nicotinic Ac.				Gln	
Local *vs* Systemic Leaves					
02 HAI	04 HAI	08 HAI	24 HAI	48 HAI	72 HAI
Asn	Glutarate	Val	Glucose	Asn	Pro
Butyrate	Lactate	Leu	Lactate	Ile	Butyrate
Nicotinic Ac.	Succinate	Ile	Butyrate	Homoserine	Val
Asp	Glycolate	Gln	Nicotinic Ac.	Gln	Glu
Citrate	Nicotinic Ac.	Asp	Ala	Leu	Glutarate
Leu	Ala	Butyrate	Salicylic Ac.	Thr	Nicotinic Ac.
Ser	Ser	Malonate	Gly	Malonate	Glucose
Lactate	Pyruvate	Ala	Leu	Butyrate	Fumarate
Gly	Glu	Homoserine	Homoserine	Glu	Citrate
Oxalate		Pyruvate	Glycerate	Fumarate	Ile
Fumarate		Glucoheptulose	Glycolate	Val	Ala
		Nicotinic Ac.	Glycerol	Nicotinic Ac.	Leu
		Gly	Fumarate		Homoserine
					Malonate
					Gln

Table S2. Number of features detected by liquid chromatography mass spectrometry (LC/MS) that were significantly altered over time or after CPSMV (cowpea severe mosaic virus) inoculation. The data from LC/MS-based metabolic fingerprinting analysis refers to the intensity of the features identified in local and systemic leaves infected with Mock (control group) or CPSMV. The pattern of accumulation of the features over time was analysed by analysis of variance (ANOVA) ($P < 0.05$) using the entire data set (all time points) of local and systemic leaves infected with Mock or CPSMV. Features with no changes by CPSMV infection are identified with ns (non-significant). This statistical analysis was carried out using the Metaboanalyst platform.

A) Metabolic fingerprinting (raw data)		
	Local	**Systemic**
ANOVA - Time	0	0

B) Metabolic fingerprinting (CPSMV/Mock ratio)		
	Local	**Systemic**
ANOVA – Time	34	35

C) Metabolic fingerprinting (CPSMV vs Mock comparison)
02h – ns
04h – ns
08h – 1 feature different between CPSMV and Mock
24h – ns
48h – ns
72h – ns

Table S3. List of metabolites identified by liquid chromatography mass spectrometry (LC/MS) analysis in local and systemic leaves infected with Mock (control group) or CPSMV (cowpea severe mosaic virus). The pattern of accumulation of the metabolites over time was analysed by analysis of variance (ANOVA) ($P < 0.05$) using raw and normalized data of local and systemic leaves infected with Mock or CPSMV. Raw values refer to the level of the metabolites normalized by the internal control and the fresh weight used in the extraction, while the CPSMV/Mock ratio refer to the values observed in CPSMV-treated leaves normalized with the mock treatment in each time point. Metabolites with no changes over time are identified with ns (non-significant). This analysis was carried out using the Metaboanalyst platform.

	Raw data		**CPSMV/Mock ratio**	
Metabolites	**Local**	**Systemic**	**Local**	**Systemic**
(3Z)-Phytochromobilin	ns	ns	ns	ns
(S)-10,16-Dihydroxyhexadecanoic acid	ns	ns	ns	ns
1,3,7-Trimethyluric acid	ns	ns	ns	ns
2,5-Diamino-6-(5'-phosphoribosylamino)-4-pyrimidineone	ns	ns	ns	ns
2-Dehydro-3-deoxy-D-arabino-heptonate 7-phosphate	ns	ns	ns	ns
2-Oxo-3-hydroxy-4-phosphobutanoic acid	ns	ns	ns	ns
2-Succinylbenzoate	ns	ns	ns	ns
4,5-seco-Dopa	ns	ns	ns	ns
4-Amino-4-deoxychorismate	ns	ns	ns	ns
4-Coumaryl alcohol	ns	ns	ns	ns
4-Hydroxycinnamyl alcohol 4-D-glucoside	ns	ns	ns	ns
4-Methyl-5-(2-phosphoethyl)-thiazole	ns	ns	ns	ns
4-Phosphopantothenoylcysteine	ns	ns	ns	ns
5,10-Methylene-THF	ns	ns	ns	ns
5-Amino-6-(5'-phosphoribitylamino)uracil	ns	ns	ns	ns
5-Hydroxyconiferyl alcohol	ns	ns	ns	ns
5-Hydroxykynurenine	ns	ns	ns	ns
5-O-(1-Carboxyvinyl)-3-phosphoshikimate	ns	ns	ns	ns
7-Methylthioheptyl glucosinolate	ns	ns	ns	ns
8'-Hydroxyabscisate	ns	ns	ns	ns
8-Methylthiooctyl glucosinolate	ns	ns	ns	ns
8-Methylthiooctyl-desulfoglucosinolate	ns	ns	ns	ns
AICAR	ns	ns	ns	ns
Biliverdin	ns	ns	$P < 0.05$	ns
Caffeyl alcohol	ns	ns	ns	ns
Caprylic acid	ns	ns	ns	ns

CDP	ns	ns	ns	ns
CDP-Ethanolamine	ns	ns	ns	ns
Chitobiose	ns	ns	ns	ns
Chlorophyll b	ns	ns	ns	ns
Cinnamaldehyde	ns	ns	ns	ns
Citicoline	ns	ns	ns	ns
Coproporphyrin III	ns	ns	ns	ns
Cyanidin 3-glucoside	ns	ns	ns	ns
Cyanidin 3-O-beta-D-sambubioside	ns	ns	ns	ns
Cyanin	ns	ns	ns	ns
Decanoyl-CoA (n-C10:0CoA)	ns	ns	ns	ns
Delphinidin 3-O-beta-D-glucoside	ns	ns	ns	ns
Deoxyadenosine	ns	ns	ns	$P < 0.05$
Deoxyuridine	ns	ns	ns	ns
Digalacturonic acid	ns	ns	ns	ns
Dihydrofolic acid	ns	ns	ns	ns
Divinylprotochlorophyllide	ns	ns	ns	ns
Dolichyl diphosphate	ns	ns	ns	ns
Dopamine	ns	ns	ns	ns
D-Sedoheptulose 7-phosphate	ns	ns	ns	ns
D-Xylitol	ns	ns	ns	ns
FAD	ns	ns	ns	ns
Flavin Mononucleotide	ns	ns	ns	ns
Galactosylglycerol	ns	ns	ns	ns
gamma-Glutamylcysteine	ns	ns	ns	ns
Geranyl 2-methylbutyrate	ns	ns	ns	ns
Gibberellin A34-catabolite	ns	ns	ns	ns
Glycineamideribotide	ns	ns	ns	ns
Guanosine	ns	ns	ns	ns
Guanosine diphosphate	ns	ns	ns	ns
Heme O	ns	ns	ns	ns
Homoeriodictyol chalcone	ns	ns	ns	ns
Hydroxymethylbilane	ns	ns	ns	ns
Hypoxanthine	ns	ns	ns	ns
Isopentenyladenosine-5'-triphosphate	ns	ns	ns	ns
Kaempferol 3-O-rhamnoside-7-O-glucoside	ns	ns	ns	ns
L-Malic acid	ns	ns	ns	ns
L-Tryptophan	ns	ns	ns	ns
Magnesium protoporphyrin	ns	ns	ns	ns
Magnesium protoporphyrin monomethyl ester	ns	ns	ns	ns
Melibiitol	ns	ns	ns	ns
Methyl jasmonate	ns	ns	ns	ns

Myricetin	ns	ns	ns	ns
Octanoyl-CoA	ns	ns	ns	ns
Ophthalmic acid	ns	ns	ns	ns
Oxidized glutathione	ns	ns	ns	ns
Pantetheine	ns	ns	ns	ns
Pelargonidin 3-O-beta-D-sambubioside	ns	ns	ns	ns
Phosphorylcholine	ns	ns	ns	ns
Phytyl diphosphate	ns	ns	ns	ns
Pinobanksin	ns	ns	ns	ns
Precorrin 2	ns	ns	ns	ns
Primary fluorescent chlorophyll catabolite	ns	ns	ns	ns
Protoporphyrin IX	ns	ns	ns	ns
Pyridoxine 5'-phosphate	ns	ns	ns	ns
Quercetin 3-glucoside	ns	ns	ns	ns
Quercetin 3-O-rhamnoside 7-O-glucoside	ns	ns	$P < 0.05$	ns
Riboflavin	ns	ns	ns	ns
S-Adenosylhomocysteine	ns	ns	ns	ns
Secologanin	ns	ns	ns	ns
Sinapic acid	ns	ns	ns	ns
Sinapine	ns	ns	ns	ns
Sinapyl alcohol	ns	ns	ns	ns
Stachyose	ns	ns	ns	ns
Syringin	ns	ns	ns	ns
Tetrahydrofolyl-[Glu](n)	ns	ns	ns	ns
Thiamine	ns	ns	ns	ns
Thiamine monophosphate	ns	ns	ns	ns
Uridine	ns	ns	ns	ns
Xanthine	ns	ns	ns	ns
Xanthylic acid	ns	ns	ns	ns

3 CHAPTER II: THE COWPEA RESISTANCE TO VIRUS INFECTION IS ASSOCIATED TO HIGHER GLYCOLYTIC FLUXES TOWARD SHIKIMATE SYNTHESIS

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Running tittle: On the metabolic basis underpinning virus resistance

Highlight: We unveiled the metabolic pathways associated to virus infection by comparing sensitive and resistant cowpea genotypes. Our results suggest that virus resistance involves a greater glycolytic flux toward shikimate synthesis.

3.1 Abstract

Cowpea is an important crop that has high tolerance to environmental stress conditions. However, cowpea severe mosaic virus (CPSMV) infection can lead to severe yield losses. Although certain cowpea genotypes are intriguingly resistant to CPSMV, they have very low yield. By contrast, high productivity cowpea genotypes are sensitive to CPSMV infection. It is thus important to understand how cowpea genotypes overcome CPSMV infection and which mechanisms regulate the growth-virus tolerance trade-off. Here we characterized a resistant (macaibo) and a susceptible (pitiúba) cowpea genotype to CPSMV infection by different metabolomics approaches. The metabolic profile of macaibo and pitiúba differed substantially even in the absence of CPSMV infection. Pitiúba showed higher content of sugars in the absence of CPSMV infection and a higher capacity to absorb glucose, which might be related to its higher growth capacity. ^{13}C-glucose labelling experiment highlights that the glucose-derived ^{13}C distribution throughout primary metabolism is very different between the genotypes. Although the level of shikimate was higher in pitiúba in the absence of stress, only macaibo showed increased content in this metabolite after CPSMV infection. Furthermore, the relative ^{13}C-enrichment in shikimate was substantially higher in macaibo than pitiúba over time of CPSMV infection. Our study unveiled the metabolic basis of cowpea CPSMV resistance, which likely involves a higher use of glycolytic carbons toward shikimate synthesis.

Key words: ^{13}C-labelling analysis, metabolomics, TCA cycle, virus resistance.

3.2 Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is a leguminous crop belonging to the Fabaceae family, which includes other important crops such as peas, beans, chickpeas, peanuts, lentils and soybeans. Cowpea is produced mainly in semi-arid climate regions, which is due to its high tolerance to abiotic stress conditions such as low water availability, high temperatures and saline soils. The cultivation of cowpea in these regions is further associated to the low production cost and the high nutritious value of its grains, making this an important source of proteins and carbohydrates for the population of these regions (ADEGBITE; AMUSA, 2010; GONÇALVES et al., 2013; KAREEM; TAIWO, 2007; SINGH et al., 2011). However, despite their high resilience to adverse environmental conditions, diseases caused by viruses currently represents the major cause of yield loss in cowpea (PAIVA et al., 2016; SOUZA et al., 2017). It is thus important to understand how cowpea plants respond to virus infection, in order to maintain the productivity of this important crop.

About twenty species of viruses have been identified as causing diseases in cowpea, including the cowpea severe mosaic virus (CPSMV), that have been leads to severe yield losses in cowpea (LIMA, 2015; LIMA et al., 2005). CPSMV belongs to the genus Comovirus (Secoviridae) and as such, causes diseases mainly in leguminous plants (BRUENING; LOMONOSSOFF, 2011; CRUZ; ARAGÃO, 2014). CPSMV thus represents a risk not only for cowpea but also for Fabaceae plants. The disease caused by CPSMV have clear symptoms such as yellowish spots on the leaves, which is result of chlorosis that occurs in the infection region. In more advanced cases of the infection, it can be observed wrinkling, leaf deformation, dwarfism and even the death of the plant (BRUENING; LOMONOSSOFF, 2011; SILVA et al., 2016a). CPSMV is an intracellular biotrophic pathogen, like all viruses, which makes it difficult to control in a large-scale cultivation. However, plants have acquired not only signalling pathways and communication mechanism that aid them to acclimate or avoid virus infection, but certain genotypes have developed mechanisms that leads to an incompatible interaction with the virus, i.e. the virus can infect the plant but few to no symptoms are observed in the plant (HOOKS; FERERES, 2006; NICAISE, 2014). Among these virus-defensive mechanisms, the effector-triggered immunity (ETI) and the systemic acquired resistance (SAR) greatly aid plants to fight against viruses and other pathogens.

The ETI can modulates the amount of phytohormones produced and activates local defensive mechanisms, aiming to reduce the vulnerability of local tissues to the attack of

the pathogen. Simultaneously, signalling molecules produced in the area of infection are transmitted to non-infected organs of the same plant through the vasculature system and/or by volatiles, as part of the SAR mechanism (GILROY et al., 2014). The volatiles, which are organic compounds mostly composed by secondary metabolites, can further inform other plants about the pathogen presence (JING et al., 2021; JOHNSON; GILBERT, 2015). Furthermore, both local and systemic responses may involve changes in the level of primary metabolites and their key metabolic networks, such as the tricarboxylic acid (TCA) cycle (MACLEAN; LEGENDRE; APPANNA, 2023; NUNES-NESI et al., 2008). This pathway is an important hub and provides substrate for the energetic metabolism and defence-related pathways (NICAISE, 2014; ZANDALINAS et al., 2022; ZANDALINAS; MITTLER, 2022). However, although evidence indicates that viral infection can substantially alters the plant metabolism (ISLAM et al., 2019; MAUCK; KENNEY; CHESNAIS, 2019), little is known concerning how primary metabolites and the TCA cycle are involved in the defence against viruses.

Evidence suggests that the metabolic changes induced by viral infection are pathosystem-dependent and cannot be generalized based on a model pathosystem (ISLAM et al., 2019; LLAVE, 2016). Previous studies carried out using resistant or susceptible cowpea genotypes to CPSMV have observed alterations in gene expression, proteomic profile, the activities of classic plant defence enzymes and accumulation of ROS, among others (LIMA et al., 2005; LIMA, 2015; MAGALHÃES, 2011; SILVA et al., 2016a; SOUZA et al., 2017; VARELA et al., 2017, 2019). Despite these advances in the study of the cowpea/CPSMV pathosystem, no studies have unveiled the metabolic basis that modulates compatible and incompatible interactions between cowpea genotypes and CPSMV. It is thus currently unclear which metabolic pathways are associated to CPSMV resistance, hampering our understanding on how cowpea plants respond to CPSMV infection. In this context, we have recently demonstrated that CPSMV infection substantially altered the dynamic of primary metabolites of the CPSMV-resistant macaibo genotype, with little impact on the secondary metabolism (BRET et al., 2023). However, the lack of metabolic data from CPSMV-susceptible genotypes makes difficult to conclude which metabolic pathways are associated to the macaibo CPSMV resistance. Here, we have characterized the CPSMV-induced metabolic responses of macaibo (resistant) and pitiúba (susceptible) cowpea genotypes by using two metabolomics approaches, namely metabolite profiling and ^{13}C-labelling analyses. Based in our previous results, we hypothesized that metabolic pathways associated to the balance

between organic acids and amino acids could be associated to the CPSMV resistance of macaibo, when compared to pitiúba.

3.3 Material and Methods

3.3.1 Experimental set up

We harvested leaves directly infected with CPSMV (herein called local leaves) and those from the same leaflet (herein called systemic leaves). Our previous results indicate that the major CPSMV-induced metabolic changes in macaibo leaves under CPSMV were observed at 08 and 48 hours after CPSMV inoculation (HAI) (BRET et al., 2023). We thus initially performed an experiment harvesting local and systemic macaibo and pitiúba leaves at 08 and 48 HAI. A second experiment was carried out to investigate how CPSMV inoculation differentially affects the metabolic fluxes in macaibo and pitiúba leaves. Leaf disks from both genotypes were obtained from local leaves inoculated with mock or CPSMV, immersed in a solution containing ^{13}C-glucose, and harvested after 0, 2, 4 and 8 h under this condition.

3.3.2 Plant material and growth conditions

We used two cowpea (*Vigna unguiculata* (L.) Walp.) genotypes, namely macaibo and pitiúba, which are resistant and sensitive to CPSMV infection, respectively (LIMA et al., 2011). Seeds were disinfected by adding 0.05% sodium hypochlorite, washed in distilled water and germinated in filter paper rolls (28x38 cm; Germitest), soaked with distilled water (1:2, m/v), and kept in a growth chamber for 7 days with a 12 h photoperiod and 50 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The seedlings were transferred to 6 L plastic pots (6 seedlings per pot) and maintained under hydroponic system using Hoagland and Arnon's nutrient solution, with slight modifications (HOAGLAND; ARNON, 1950; SILVEIRA; COSTA; OLIVEIRA, 2001). The solution was completely changed once a week. Plants were grown under greenhouse conditions with natural sunlight (300-650 µmoles m^{-2} s^{-1} PPFD), daily temperature ranging from 27 to 31.0 °C and 79.8 ± 10.9% of average relative humidity.

3.3.3 CPSMV inoculation and harvest of leaf material

The inoculum of cowpea severe mosaic virus (CPSMV), strain CE, was isolated from pitiúba leaves exhibiting classic symptoms of CPSMV infection, as described previously (Paz *et al.*, 1999). Virus-infected pitiúba leaves were macerated in 10 mM potassium phosphate buffer (pH 7.0) containing sodium sulfite (1:10, m/v) for 10 minutes. Carborudum (600 mesh) was added to the inoculum (1:10, m/v) to facilitate the virus penetration in leaf tissues. The inoculation of mock and CPSMV were performed on the central leaflet of the first fully expanded trifoliate by friction with thumb and index fingers. Control plants were mock inoculated using the same buffer, but without the presence of CPSMV. Local and systemic leaves were harvested after 0, 8 and 48 HAI and immediately frozen in liquid nitrogen for metabolomics analysis.

3.3.4 ^{13}C-labelling experiment

The plants were grown and inoculated as described above. Leaf disks of 0.5 cm ratio were detached from the leaflets immediately after inoculation. 12 leaf disks were immersed in a petri dish containing 24 mL of 10 mM potassium phosphate buffer (pH 7.0) and 5 mM of uniformly labeled [U^{13}C]-glucose (Sigma-Aldrich). The disks were harvested at 0, 2, 4 and 8 HAI and immediately frozen in liquid nitrogen. Each biological replicate corresponds to four leaf disks from four different plants.

3.3.5 GC-MS analysis

The extraction of polar metabolites and the GC-MS analysis were carried out as described previously (LISEC et al., 2006). Samples were derivatized by methoxyamine hydrochloride, previously dissolved in 20 mg/mL in pure pyridine, and *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (LISEC et al., 2006). Chromatograms analysis was carried out using the Xcalibur 2.1 software (Thermo Fisher Scientific). Metabolites were identified using the Golm Metabolome Database (KOPKA et al., 2005).

3.3.6 ^{13}C-enrichment analysis

The ^{13}C-enrichment in the metabolites identified from the ^{13}C-labelling experiment was determined as described previously (LIMA et al., 2018). Briefly, the fractional ^{13}C-enrichment ($F^{13}C$) in each metabolite was determined by the following equation:

$F^{13}C = ((M1 * 1)+(M2 * 2)+\ldots+(Mn * n))/n$, where **M** corresponds to the isotopologues of the mass fragment and **n** the number of carbons present in each fragment. For instance, for a metabolite fragment composed of 3 carbons, the $F^{13}C$ is obtained as following:

$F^{13}C = ((M1 * 1)+(M2 * 2)+(M3 * 3))/3$

After calculating the $F^{13}C$, we then estimated the relative ^{13}C-enrichment ($R^{13}C$) by normalizing the $F^{13}C$ obtained at 0, 2, 4 and 8 HAI according to the $F^{13}C$ of the control (time 0h) of each genotype.

3.3.7 Statistical analysis

The metabolic changes over time was evaluated using analysis of variance (ANOVA) followed by the tests of Tukey or Dunnet ($P < 0.05$). Partial least squares-discriminant analysis (PLS-DA) was carried out using the MetaboAnalyst platform (PANG et al., 2021). Punctual comparisons between mock and CPSMV treatments or between macaibo and pitiúba were carried out by Student's *t* test ($P < 0.05$). Heat maps were created using the MultiExperiment Viewer 4.9.0 software.

3.4 Results and discussion

3.4.1 The leaf metabolite profile of macaibo and pitiúba leaves is substantially different even in the absence of stress

We have previously shown that CPSMV infection substantially alter the dynamic of accumulation of primary metabolites in macaibo leaves, but had little impact on the secondary metabolism (BRET et al., 2023). Here, we aim to provide further insights on the metabolic aspects underpinning cowpea CPSMV resistance by comparing the metabolic

responses between the CPSMV-resistant macaibo with the CPSMV-sensitive pitiúba genotype. CPSMV was inoculated directly onto local leaves and these and the systemic leaves were harvested after 0, 8 and 48 hours after CPSMV inoculation (HAI). We first aimed to investigate whether the metabolite profile of macaibo and pitiúba leaves differs before the imposition of the stress. For this, we have used the partial least squares discriminant analysis (PLS-DA), that is highly effective to discriminating stressed from non-stressed plants and to unveil the metabolic pathways associated to stress conditions (CARDOSO; FREIRE; DALOSO, 2022). The comparison of leaves harvested at 0 HAI, which corresponds to 09:00 a.m. (ZT 4), showed that the metabolite profile of macaibo and pitiúba leaves differ substantially, as demonstrated by the clear separation by the first component of the PLS-DA carried out using data from either local or systemic leaves (Figures 1A-B). The variable importance in projection (VIP) from the PLS-DA highlights the metabolites that mostly contributed to the discrimination of macaibo and pitiúba leaves (Figure S1). Metabolites with VIP score higher than 1 are considered those that have greater importance for the PLSDA model (XIA; WISHART, 2011). Several of these metabolites including fumarate, glucoheptose, glutarate, shikimate, glucose, fructose and mannose have lower levels in macaibo than pitiúba in local leaves. Furthermore, lactate, phosphoric acid, glutarate, dehydroascorbate and tryptophan have lower levels while glutamate, asparagine and galactinol have higher levels in macaibo than pitiúba in systemic leaves (Figure 1C). This analysis indicates that the leaf metabolite profile of macaibo is substantially different from pitiúba in the absence of stress.

3.4.2 CPSMV inoculation alters the metabolite profile of macaibo and pitiúba leaves

We next compared the effect of CPSMV inoculation on the metabolite profile of macaibo and pitiúba leaves. Given that the metabolism of these genotypes showed several differences in the absence of stress (Figures 1A-B), we then normalized the data obtained after 08 and 48 h of mock or CPSMV inoculation according to the time 0h of each genotype. The results showed that CPSMV inoculation altered the metabolism of both macaibo and pitiúba leaves, as demonstrated by the separations between mock and CPSMV treatments by the first or second components of the PLS-DA (Figure 2). However, the CPSMV-induced metabolic changes diverge substantially between macaibo and pitiúba at local and systemic leaves, as evidenced by the clear separation by the PC1 after either 08 or 48 HAI (Figure 3).

Interestingly, all metabolites that have lower level in local macaibo leaves in the absence of stress (Figure 1), i.e. fumarate, glucoheptose, glutarate, shikimate, glucose, fructose and mannose, have higher level than local pitiúba leaves after either 08 or 48 HAI (Figure 4). Additionally, the level of pyruvate, serine, succinate and glycolate was higher in macaibo than pitiúba local leaves. By contrast, several amino acids have lower level in macaibo, when compared to local or systemic pitiúba leaves after CPSMV infection (Figure 4). Only pyruvate at 08 and 48 HAI and dehydroascorbate at 48 HAI showed higher level in macaibo than pitiúba systemic leaves (Figure 4). This analysis collectively indicates that the CPSMV-induced metabolic responses range substantially between macaibo and pitiúba leaves, in which macaibo showed higher contents of shikimate, sugars and metabolites associated to the tricarboxylic acid (TCA) cycle and the photorespiratory metabolism.

The metabolic pathways associated to sugars, organic acids and amino acids are largely documented to be associated to mechanisms that aid plants to acclimate to biotic or abiotic stress conditions (CARDOSO; FREIRE; DALOSO, 2022; FÀBREGAS; FERNIE, 2019; OBATA; FERNIE, 2012; PIRES et al., 2016; ZANDALINAS et al., 2022). Furthermore, shikimate is a key metabolite associated to plant defence against (a)biotic stresses (MAEDA; DUDAREVA, 2012; SAITO et al., 2013; TOHGE et al., 2013). As long as this metabolite appears in the VIP score list that discriminates macaibo and pitiúba CPSMV-infected local leaves (Figure S1) and have differential accumulation before and after CPSMV infection between macaibo and pitiúba (Figures 1 and 4), we next deeply investigated the dynamic of accumulation of this metabolite throughout the CPSMV infection period.

3.4.3 The content of shikimate is only altered in directly CPSMV-infected macaibo leaves

Interestingly, the level of shikimate did not change neither in local nor in systemic pitiúba leaves over time. By contrast, the level of shikimate was altered by CPSMV only in local macaibo leaves, showing an increased content at 08 HAI (Figure 5). At 48 HAI, the level of shikimate decreased to that found before the stress imposition. It is noteworthy that this dynamic of accumulation at 08 HAI and decrease at 48 HAI coincides with the level of CPSMV infection in local macaibo leaves observed previously (Bret *et al.*, 2023). However, no difference between mock and CPSMV treatments at 08 HAI was observed (Figure 5). Furthermore, changes in the level of metabolites do not necessarily correlates with changes in

metabolic fluxes (WILLIAMS et al., 2008). Taking this into account, we then next carried out a ^{13}C-labelling analysis to obtain better insights on the metabolism-mediated mechanisms associated to CPSMV resistance.

3.4.4 ^{13}C-glucose analysis highlights that pitiúba leaves have higher capacity to absorb glucose, as compared to macaibo

^{13}C-metabolic flux analyses (^{13}C-MFA) are key to unveil which metabolic pathways are associated to a determined phenotype (FERNIE; GEIGENBERGER; STITT, 2005). Plant ^{13}C-MFA have been carried out using from isolated cells, tissues and organs to the entire plant (SILVA et al., 2016b). Here, leaf disks from macaibo and pitiúba were harvested and subjected to 5 mM of uniformly ^{13}C-labeled glucose ([U^{13}C-glucose) for 0, 2, 4 and 8 h under mock or CPSMV inoculation. The metabolites were extracted, identified by GC-MS and the ^{13}C-enrichment in each metabolite was determined as described previously (LIMA et al., 2018). Intriguingly, pitiúba showed a much higher capacity to absorb glucose, as evidenced by the higher increase in the content and in the relative ^{13}C-enrichment (R^{13}C) in this metabolite, as compared to macaibo (Figure 6). However, whilst no difference between mock and CPSMV treatments in pitiúba was observed, CPSMV-inoculated leaves showed higher R^{13}C in glucose than mock-treated leaves of macaibo at 4 HAI (Figure 6). The metabolism and transport of carbohydrates are closely linked to the growth capacity of the genotype (CHEN et al., 2022; DE OLIVEIRA SILVA et al., 2018; SULPICE et al., 2009). It has been shown that the yield is much higher in pitiúba than macaibo under favorable conditions (PAIVA et al., 2014). The greater capacity to of pitiúba to absorb glucose could be due to a higher presence or activity of hexose transporters, which in turn contribute to explain, at least partially, the higher growth of this genotype, when compared to pitiúba.

3.4.5 ^{13}C-labelling analysis highlights that the glycolytic fluxes toward shikimate are higher in macaibo than pitiúba leaves under CPSMV infection

Given the differences in the capacity to incorporate glucose observed between the genotypes, we thus decided to analyse the R^{13}C data in two manners, using the raw R^{13}C data and normalized by the ^{13}C-enrichment observed into glucose (R^{13}C-Glc^{-1}) in each genotype and time point. Whilst the raw R^{13}C provides idea on the ^{13}C-incorporated in each metabolite,

the $R^{13}C\text{-}Glc^{-1}$ data better indicates the proportion of the distribution of the carbons derived from glucose throughout the metabolism (DETHLOFF; ORF; KOPKA, 2017). Analysis of variance highlights that 19 metabolites showed significant increases in $R^{13}C$ in macaibo and pitiúba leaves throughout the time after mock or CPSMV inoculation (Table S1). Among these, 15 and 19 metabolites have increased $R^{13}C$ in macaibo and pitiúba, when compared to their respective control, as indicated by analysis of variance (ANOVA) followed by the test of Dunnet (Figure 7). Only isoleucine, succinate, fumarate and citrate did not show increased $R^{13}C$ in macaibo after mock or CPSMV inoculation. Several of these metabolites showed higher $R^{13}C$ in pitiúba than macaibo, which is rather than expected given the higher incorporation of ^{13}C-glucose in pitiúba (Figure 7). However, this trend is reversed in the $R^{13}C\text{-}Glc^{-1}$ data, in which macaibo showed higher ^{13}C-enrichment in almost all metabolites, with exception of isoleucine (Figure 8).

PLS-DA using the $R^{13}C\text{-}Glc^{-1}$ data indicates that the glucose-derived ^{13}C-distribution throughout the metabolism is very different between macaibo and pitiúba, as indicated by the separation of both genotypes by the PC1, despite the inoculation of mock or CPSMV (Figure 9). Shikimate, glutamate, GABA, *myo*-inositol and metabolites associated to the TCA cycle (2-oxoglutarate, citrate, pyruvate) and the photorespiratory (glycine, serine, glycerate) metabolism have VIP score higher than 1 (Figure S2), being thus the metabolites that mostly contributed to the discrimination of macaibo and pitiúba leaves. Given that several of these metabolites have been previously reported as biomarkers for different stress conditions (CARDOSO; FREIRE; DALOSO, 2022; CHOUDHURY et al., 2018; FÀBREGAS; FERNIE, 2019; MACLEAN; LEGENDRE; APPANNA, 2023; OBATA; FERNIE, 2012; ZANDALINAS; MITTLER, 2022) and have been found in the VIP score list of the first experiment (Figure S1), we next deeply investigated the dynamic of the $R^{13}C\text{-}Glc^{-1}$ in some of them throughout the time of mock or CPSMV inoculation in each genotype.

3.4.6 CPSMV inoculation reduce the metabolic fluxes from pyruvate to metabolites of, or associated to, the TCA cycle

The TCA cycle is an important hub for the plant growth-stress tolerance trade-off, given that it can provide substrates for the synthesis of growth-related pathways such as those associated to the (photo)respiratory metabolism and the assimilation of nitrogen as well as to the synthesis of phytohormones and secondary metabolites, which are key for growth and

defence, respectively (ARAÚJO et al., 2012; DE SOUZA et al., 2020; FERNIE; CARRARI; SWEETLOVE, 2004; MØLLER et al., 2020). The TCA cycle can be fed with different substrates, from pyruvate to amino acids (LE; MILLAR, 2022). Pyruvate is the ending product of glycolysis, being a hub that connects the catabolism of sugars with the TCA cycle (LE; LEE; MILLAR, 2021). Interestingly, the $R^{13}C$ in pyruvate is higher in macaibo than pitiúba but had no difference between mock or CPSMV treatments in any genotype. This result indicates that the glycolytic fluxes toward pyruvate are higher in macaibo but are not altered by CPSMV inoculation. However, the $R^{13}C\text{-}Glc^{-1}$ in the TCA cycle metabolites citrate, 2-oxoglutarate, and malate is higher in mock than CPSMV-treated leaves in macaibo, while no difference in the $R^{13}C\text{-}Glc^{-1}$ in these metabolites was observed in pitiúba (Figure S3). Furthermore, the $R^{13}C\text{-}Glc^{-1}$ in metabolites that depends on carbon from the TCA cycle such as aspartate, GABA and glutamate was lower in the presence of CPSMV in macaibo, when compared to the mock treatment in this genotype, and this was not observed in pitiúba (Figure S4). These results suggest that the conversion from pyruvate to metabolites of, or associated to, the TCA cycle are inhibited by the presence of CPSMV in macaibo, but not in pitiúba.

3.4.7 The glycolytic fluxes toward shikimate are higher in macaibo than pitiúba leaves

The ^{13}C-enrichment in shikimate was much higher in macaibo than pitiúba leaves, especially when the data is normalization by the ^{13}C-enrichment in glucose (Figure 10). Both $R^{13}C$ and $R^{13}C\text{-}Glc^{-1}$ in shikimate were not altered by CPSMV in pitiúba. However, while no difference in the $R^{13}C$ in shikimate between mock and CPSMV treatments was observed, mock-treated leaves have higher $R^{13}C\text{-}Glc^{-1}$ in this metabolite, when compared to CPSMV-treated leaves in macaibo. These results highlight that macaibo has higher constitutive glycolytic fluxes toward shikimate synthesis than pitiúba, but the presence of CPSMV did not increase these fluxes toward shikimate in any genotype. Given the role of the shikimate pathway for stress tolerance (MAEDA; DUDAREVA, 2012) and the fact that the macaibo resistance to CPSMV may involve constitutive mechanisms, i.e. not only responses that are activated upon CPSMV infection, we hypothesize that a higher constitutive flux toward shikimate synthesis may aid macaibo leaves to struggle against CPSMV infection.

Evidence highlights that the CPSMV inoculation substantially increased ROS accumulation in leaves of both macaibo and pitiúba (MAGALHÃES, 2011). In this context,

shikimate is substrate for the synthesis of a wide range of ROS-scavenging compounds such as polyphenols (TOHGE et al., 2013). Thus, higher glycolytic fluxes toward shikimate may contribute to avoid oxidative stress in macaibo leaves upon CPSMV infection. Indeed, previous results indicate that the reduction in the level of ROS over time was associated with an increase in the activity of the phenylalanine ammonia-lyase (PAL), an enzyme of the shikimate pathway. Interestingly, the activity of PAL was higher in CPSMV than mock-inoculated macaibo leaves, while the opposite was observed in pitiúba leaves at 12 HAI (MAGALHÃES, 2011). PAL is a key enzyme for the synthesis of defense-related compounds such as phenylpropanoids and the salicylic acid (SA), which leads to SAR in several plant species (GURIKAR et al., 2022; TOHGE; DE SOUZA; FERNIE, 2017).

Our results collectively highlight that the metabolic responses to CPSMV infection differ substantially between macaibo (resistant) and pitiúba (susceptible) genotypes. The lower ^{13}C-enrichment in TCA cycle metabolites in local macaibo CPSMV-treated leaves suggest that the metabolic fluxes from pyruvate are redirected to other pathways, rather than used by the TCA cycle. Thus, beyond a higher constitutive metabolic flux toward shikimate, other metabolic pathways that depends on carbon from pyruvate could be associated to the resistance of CPSMV in macaibo. Further studies are then necessary to obtain further insights into the metabolism-mediated mechanisms the regulate CPSMV resistance in cowpea.

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3.6 Figures Chapter II

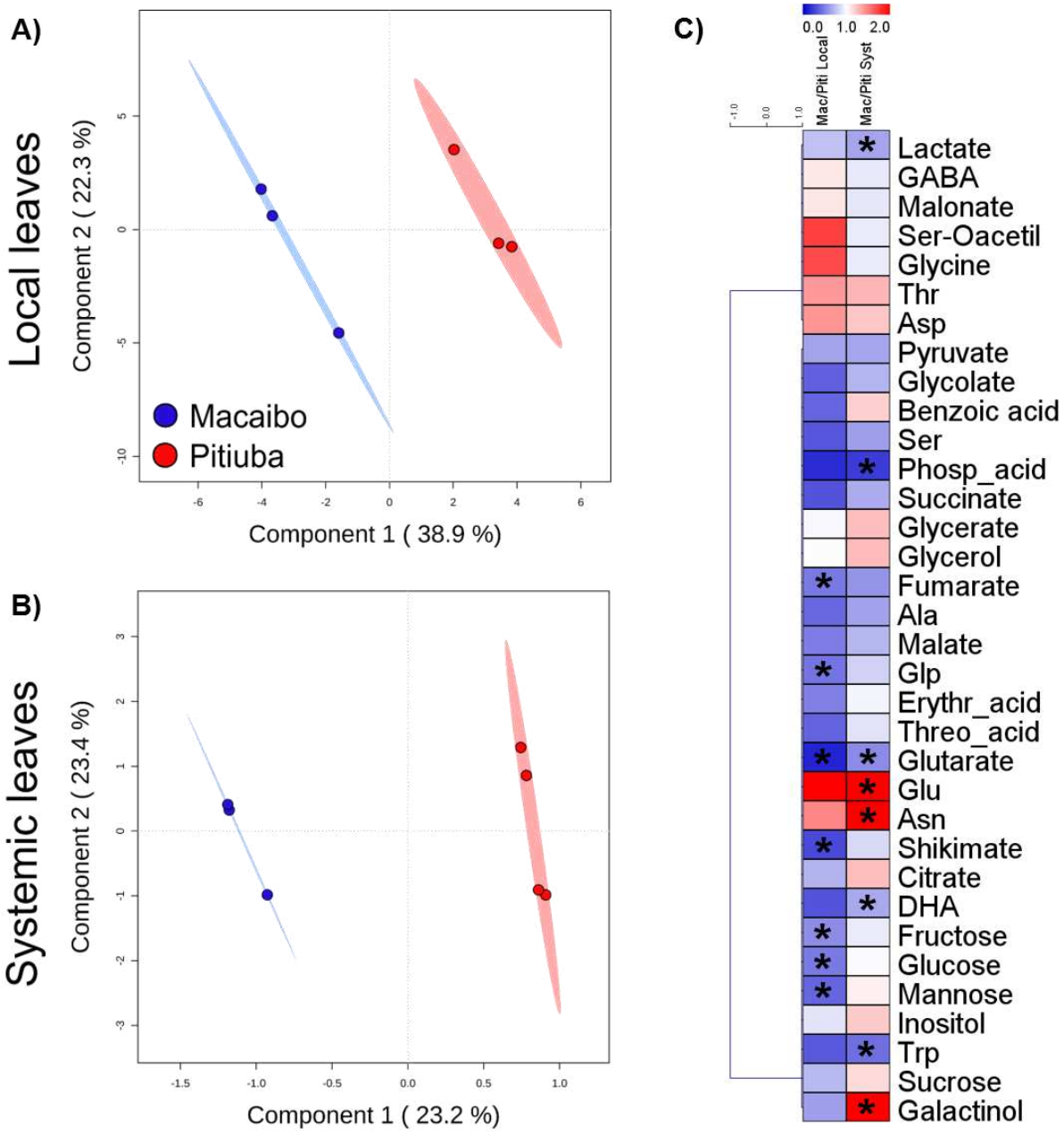

Figure 1. Partial least squares discrimination analysis (PLS-DA) and heatmap representation of the metabolite profiling of macaibo and pitiúba leaves. A-B) PLS-DA was carried out using metabolite profiling data from leaves harvested at 0h, i.e. from local (A) and systemic (B) leaves harvested before the imposition of mock or cowpea severe mosaic virus (CPSMV) treatments. The percentage variation explained by the PC1 and PC2 are represented in each axis. PLS-DA was carried out using the Metaboanalyst platform. C) Heat map was built using the ratio between macaibo and pitiúba in local and systemic leaves. The data was log transformed for heat map representation. The level of each metabolite is represented by the colour scale of the legend. Red and blue squares represent metabolites with increased and decreased level in macaibo, as compared to pitiúba. Asterisks (*) indicates significant difference between macaibo and pitiúba by the Student's *t* test ($P < 0.05$).

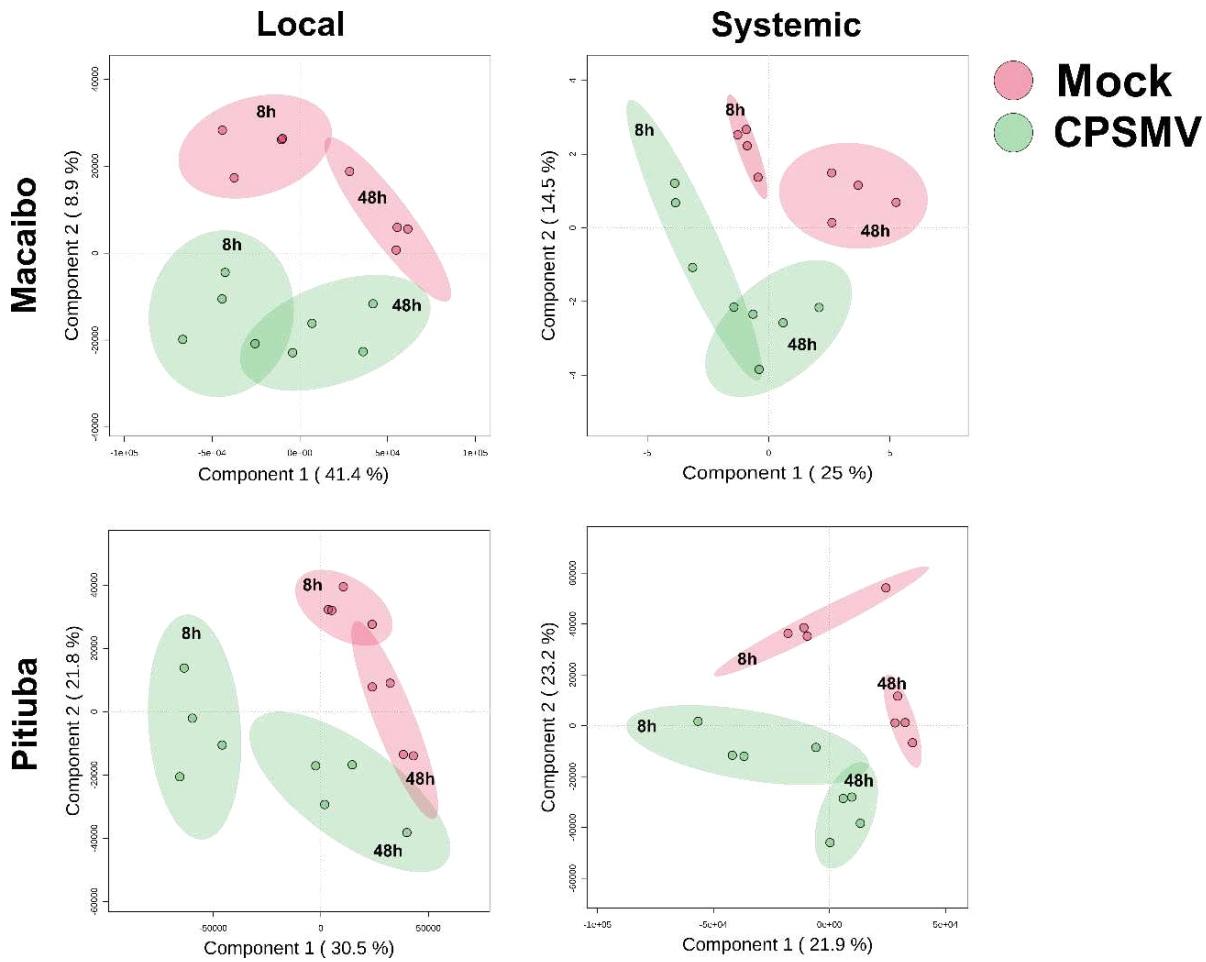

Figure 2. Partial least squares discrimination analysis (PLS-DA) of the metabolite profiling of local and systemic macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) and harvested after 08 and 48 hours. PLS-DA was carried out using the metabolite profiling data from 08 and 48 hours after mock or CPSMV inoculation relative to the time 0h of each genotype. The percentage variation explained by the PC1 and PC2 are represented in each axis. PLS-DA was carried out using the Metaboanalyst platform.

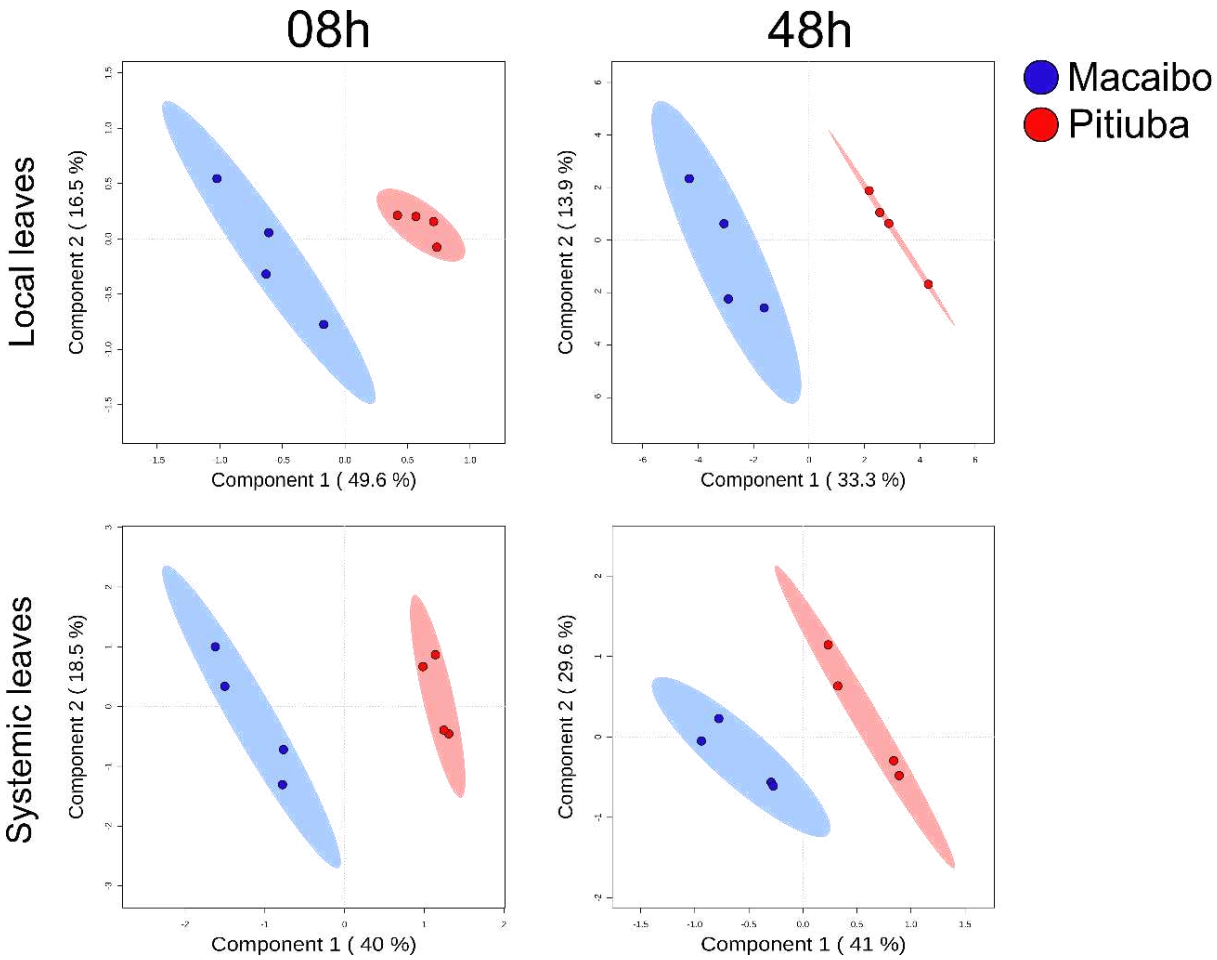

Figure 3. Punctual comparison of the metabolite profiling of local and systemic macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) and harvested after 08 and 48 hours. Partial least squares discrimination analysis (PLS-DA) was carried out using the metabolite profiling data from 08 and 48 hours after mock or CPSMV inoculation relative to the time 0h of each genotype. The percentage variation explained by the PC1 and PC2 are represented in each axis. PLS-DA was carried out using the Metaboanalyst platform.

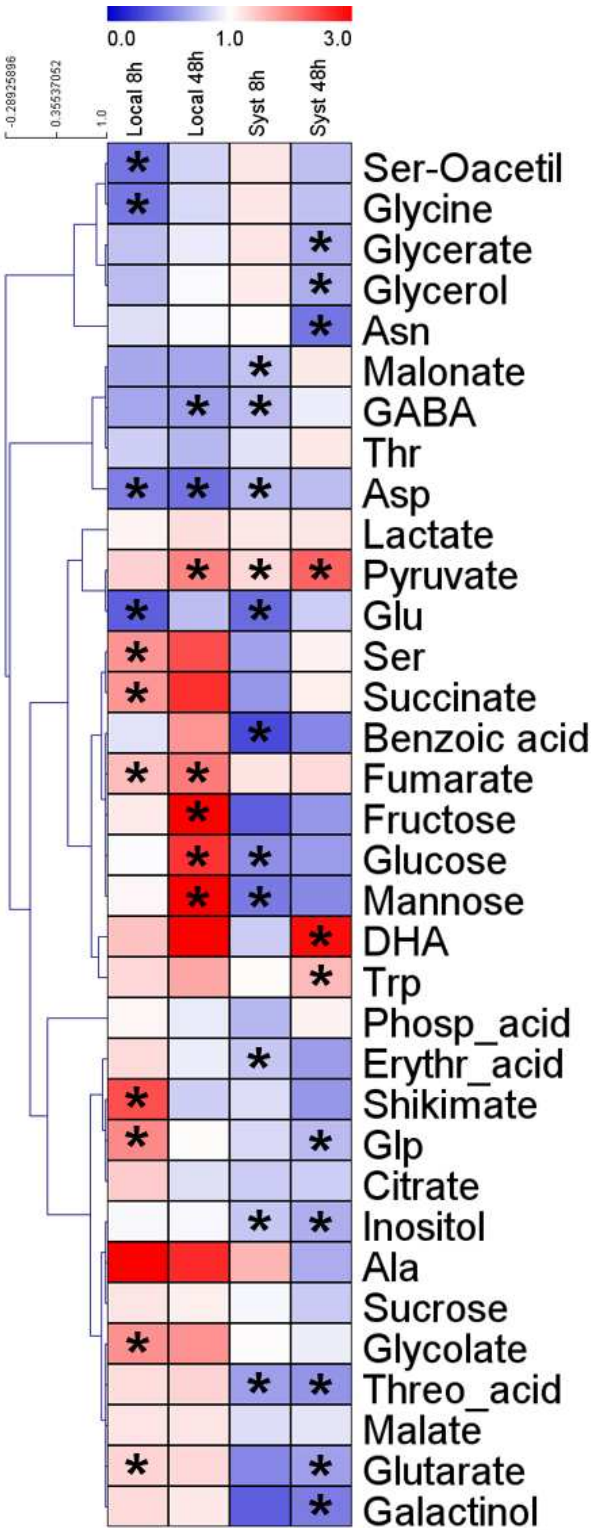

Figure 4. Heat map representation of the metabolite profiling of local and systemic macaibo and pitiúba leaves inoculated with cowpea severe mosaic virus (CPSMV) and harvested after 08 and 48 hours. The heat map was created using a log transformed metabolite profiling data relative to the time 0h of each genotype. The level of each metabolite is represented by the colour scale of the legend. Red and blue squares represent metabolites with increased and decreased level in macaibo, as compared to pitiúba. Asterisks (*) indicate significant difference between macaibo and pitiúba by the Student's *t* test ($P < 0.05$).

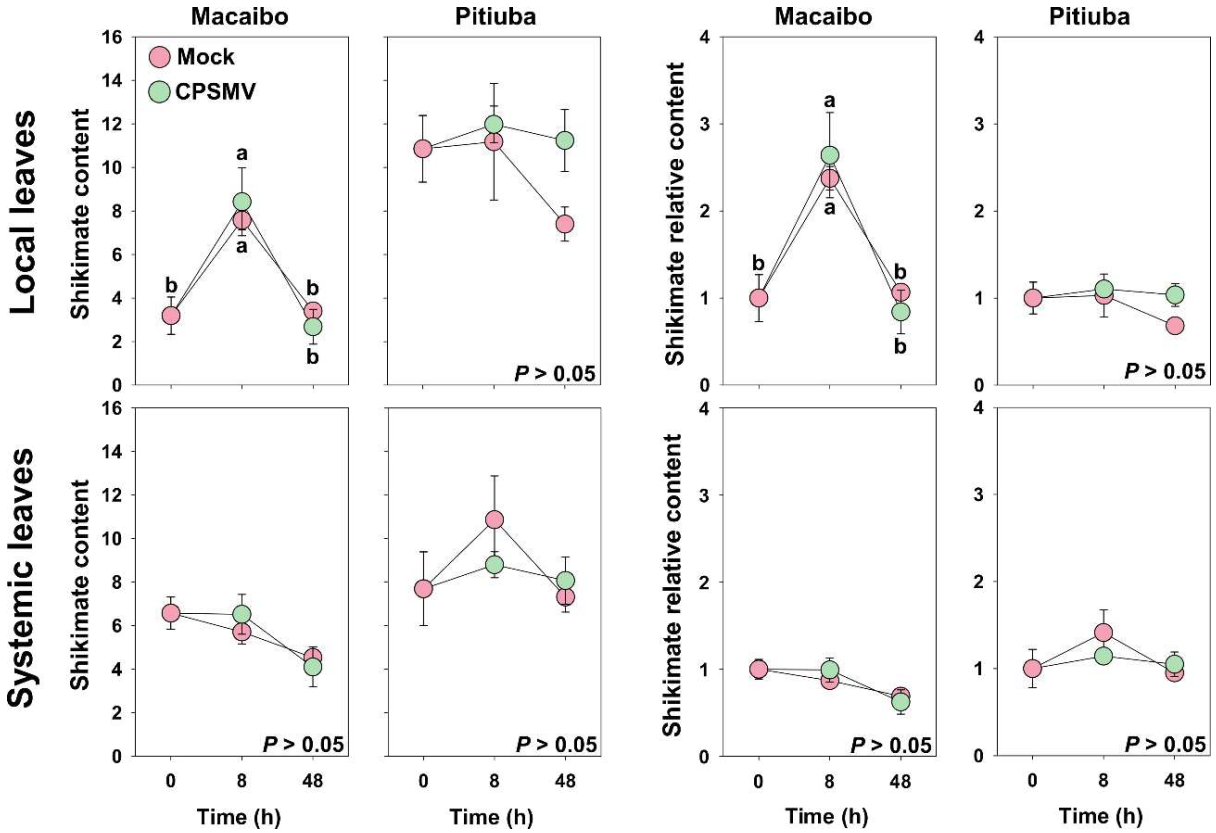

Figure 5. Shikimate content in local and systemic macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) for 0, 08 and 48 hours. Left and right figures highlight the raw metabolite content data (normalized by ribitol and the fresh weight used during the extraction) and this data normalized to the time 0h of each genotype, respectively. The level of shikimate was significantly altered only in local macaibo leaves, as indicated by analysis of variance (ANOVA) and the test of Tukey ($P < 0.05$). Values with different letters are significantly different.

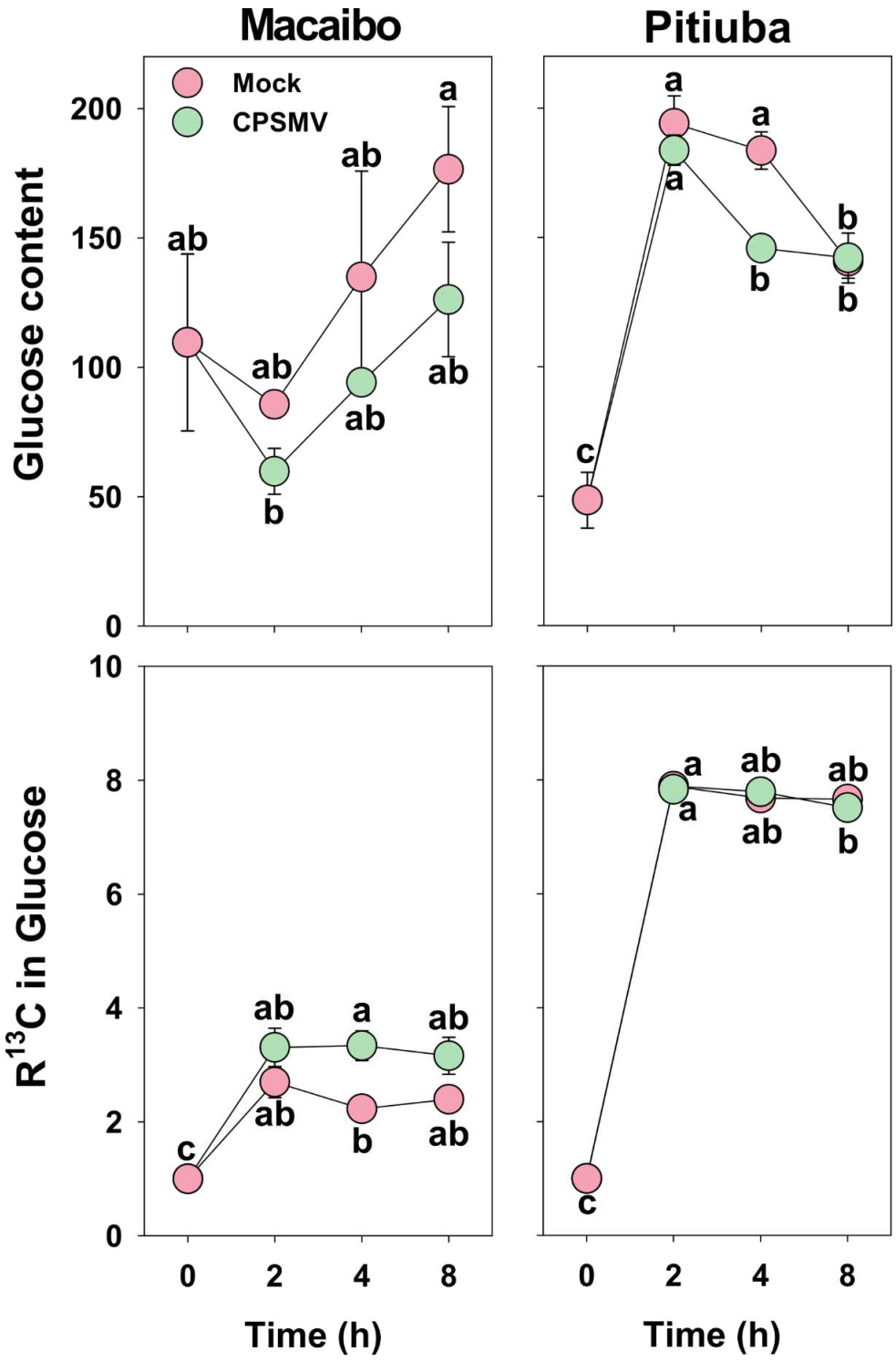

Figure 6. Changes in the content and the relative ^{13}C-enrichment ($R^{13}C$) in glucose of local and systemic macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) for 0, 2, 4 and 8 hours. Left and right figures highlight the data from macaibo and pitiúba, respectively. Values with different letters are significantly different, as indicated by analysis of variance (ANOVA) and the test of Tukey ($P < 0.05$).

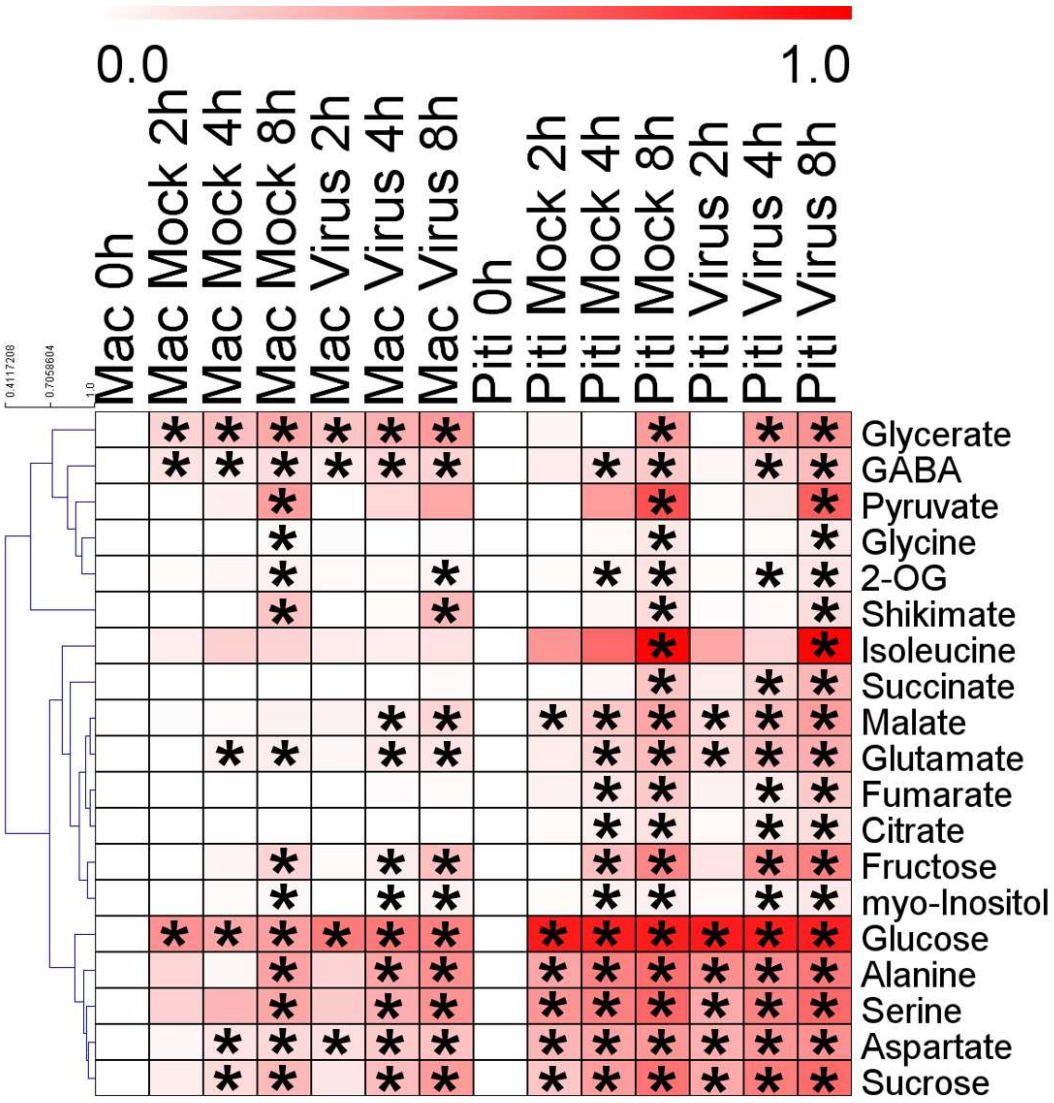

Figure 7. Heat map representation of the relative ^{13}C-enrichment ($R^{13}C$) data from macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) for 0, 2, 4 and 8 hours. The heat map was created using a log transformed $R^{13}C$ data relative to the time 0h of each genotype. Asterisks (*) indicate significant difference from the control (time 0h) of each genotype by analysis of variance (ANOVA) and the test of Dunnet ($P < 0.05$).

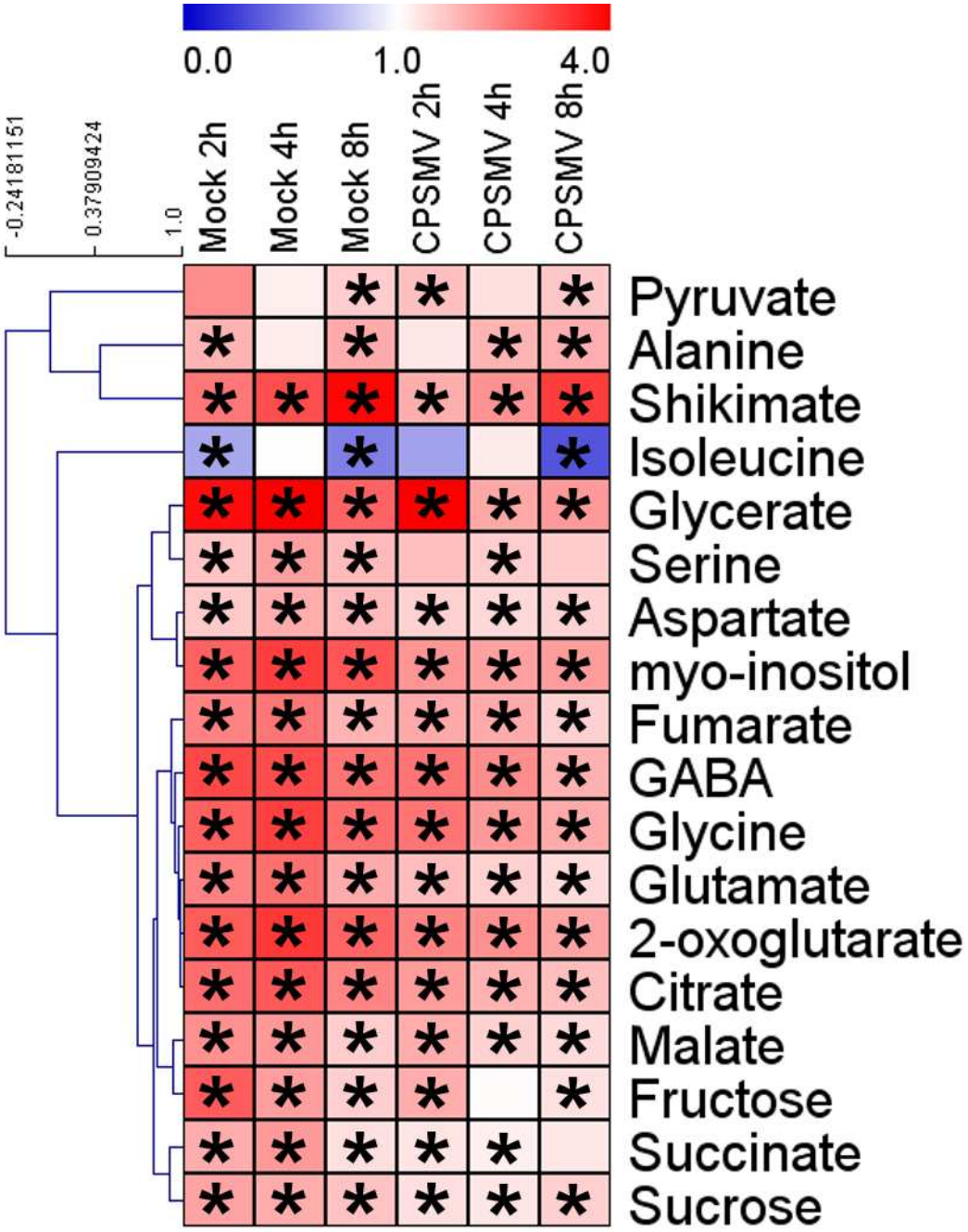

Figure 8. Heat map representation of the ratio of the ^{13}C-enrichment ($R^{13}C$) normalized by the ^{13}C-enrichment observed into glucose ($R^{13}C\text{-}Glc^{-1}$) between macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) for 2, 4 and 8 hours. The heat map was created using the macaibo to pitiúba $R^{13}C\text{-}Glc^{-1}$ ratio. Asterisks (*) indicate significant difference between macaibo and pitiúba in each time point and treatment by Student´s *t* test ($P < 0.05$).

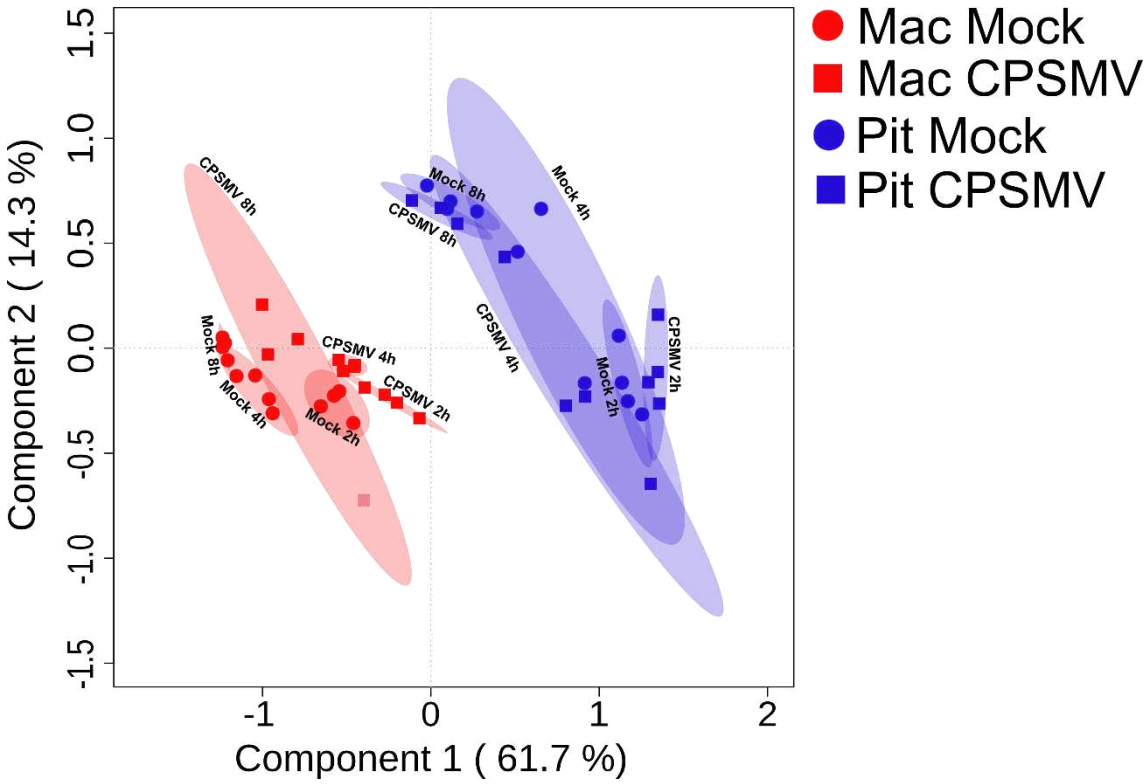

Figure 9. Partial least squares discrimination analysis (PLS-DA) of the relative ^{13}C-enrichment ($R^{13}C$) normalized according to the ^{13}C-enrichment in glucose from each genotype. The percentage variation explained by the PC1 and PC2 are represented in each axis. PLS-DA was carried out using the Metaboanalyst platform.

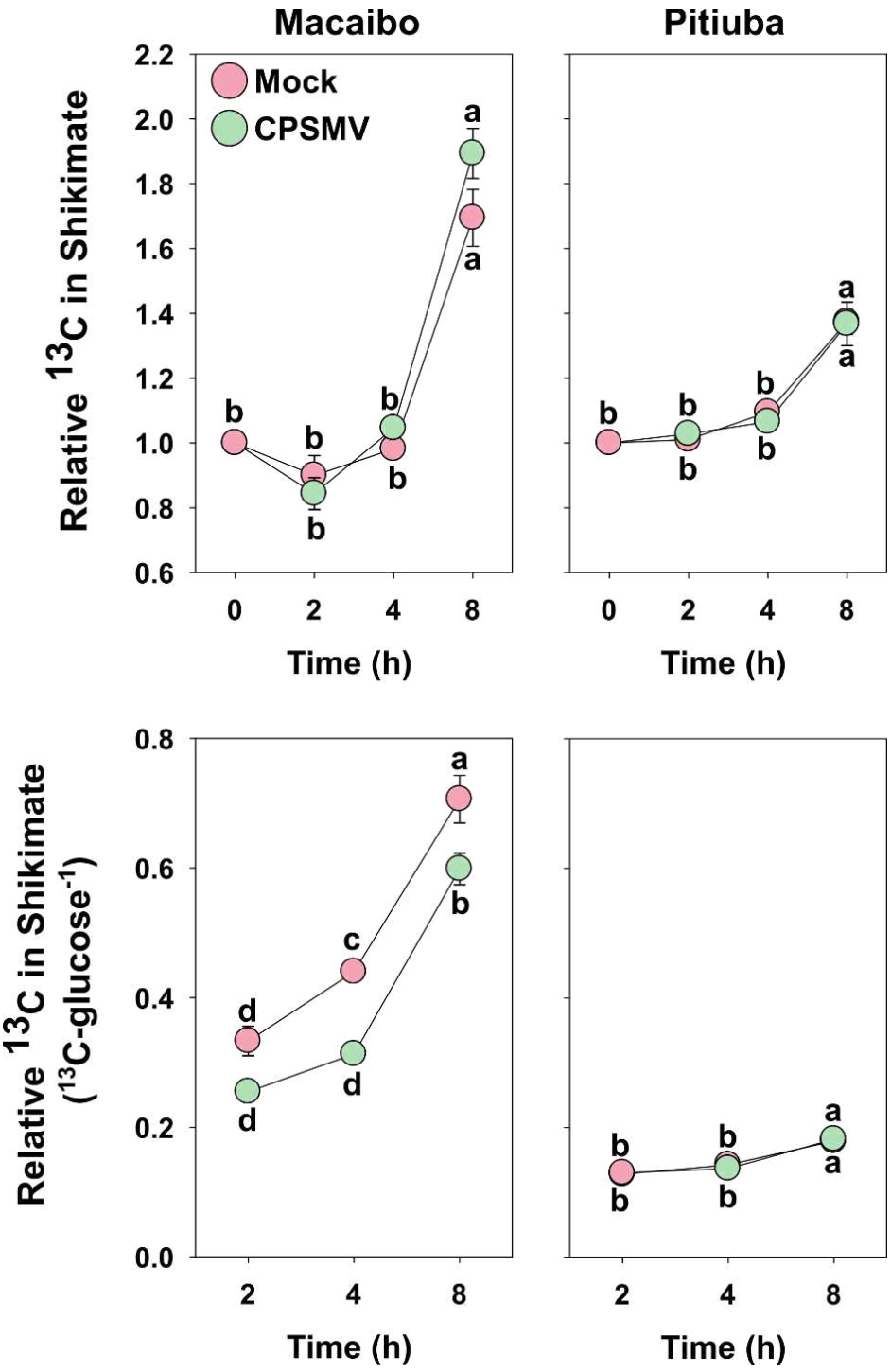

Figure 10. ^{13}C-enrichment in shikimate from macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) for 2, 4 and 8 hours. Upper and lower figures highlight the raw relative ^{13}C-enrichment ($R^{13}C$) data and the normalized according to the ^{13}C-enrichment in glucose from each genotype, respectively. Values with different letters are significantly different, as indicated by analysis of variance (ANOVA) and the test of Tukey ($P < 0.05$).

3.7 Supplemental Figures Chapter II

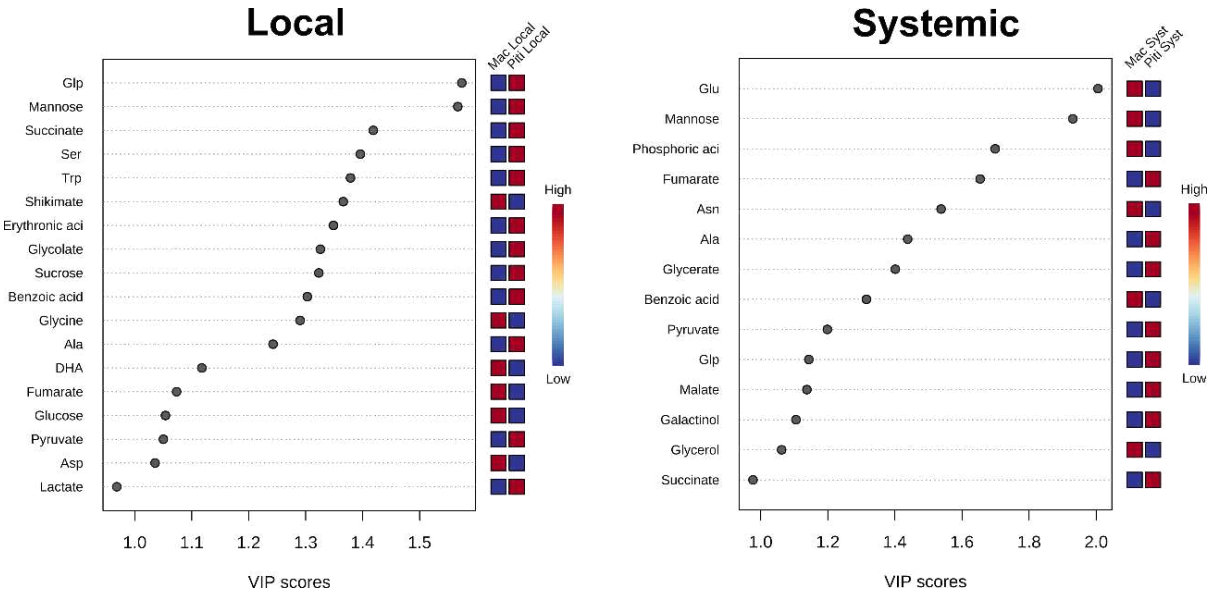

Figure S1. Variable importance in projection (VIP) from the partial least squares discriminant analysis (PLS-DA) carried out using the metabolite profiling data of local (left figure) and systemic (right figure) macaibo and pitiúba leaves. This analysis was carried out using metabolite profiling data from leaves harvested at 0h, i.e. from leaves harvested before the imposition of mock or cowpea severe mosaic virus (CPSMV) treatments. PLS-DA and VIP score list was obtained by using the Metaboanalyst platform.

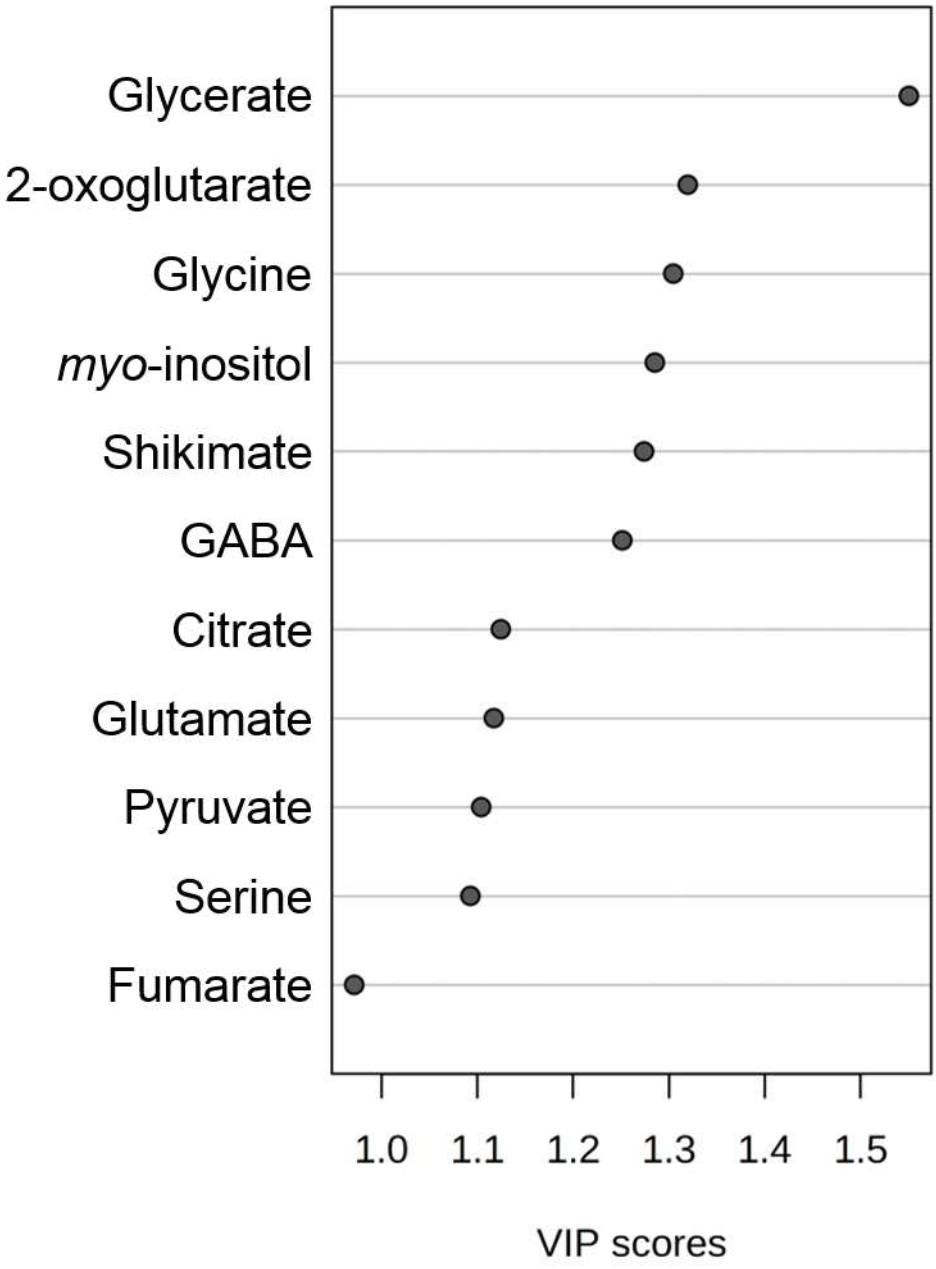

Figure S2. Variable importance in projection (VIP) from the partial least squares discriminant analysis (PLS-DA) carried out using the relative ^{13}C-enrichment ($R^{13}C$) data normalized by the ^{13}C-enrichment observed into glucose ($R^{13}C\text{-}Glc^{-1}$). PLS-DA and VIP score list was obtained by using the Metaboanalyst platform.

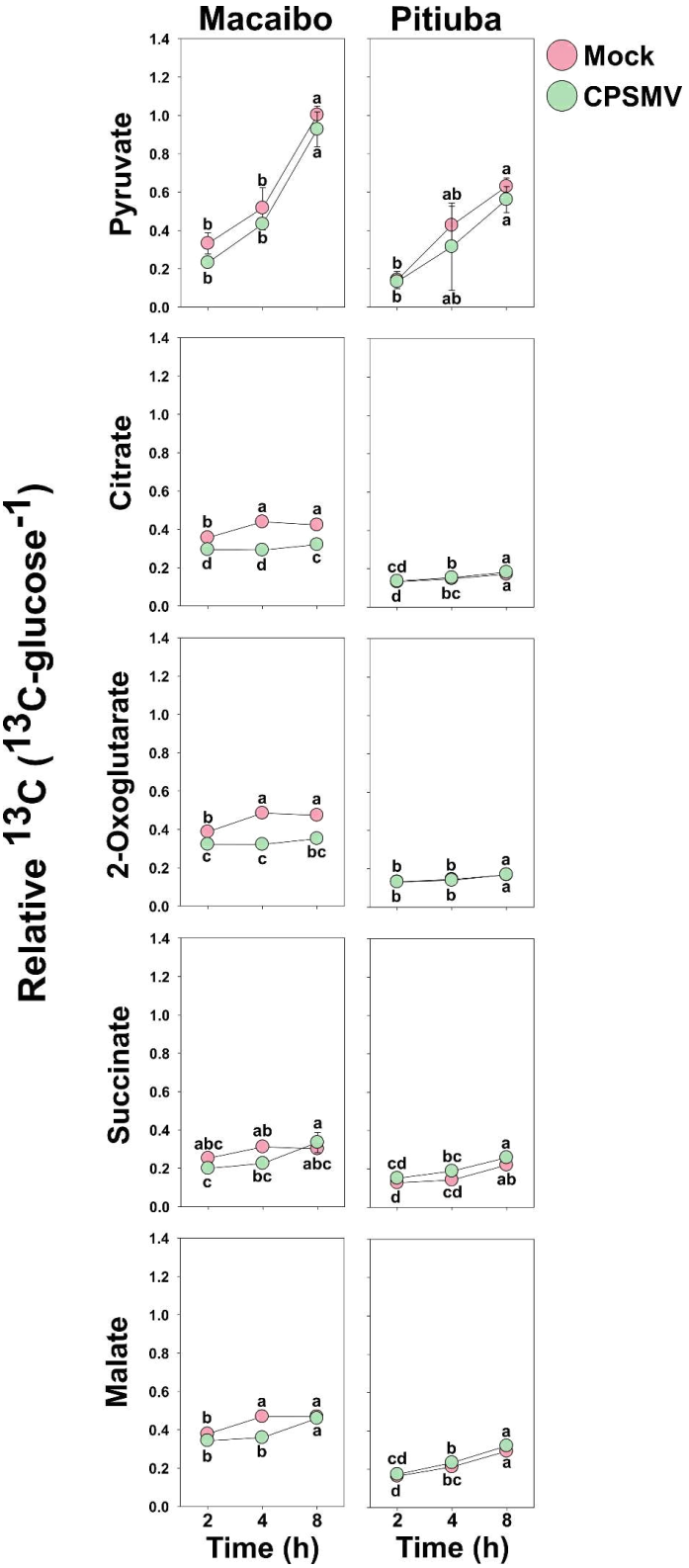

Figure S3. ^{13}C-enrichment in metabolites associated to the tricarboxylic acid (TCA) cycle in local and systemic macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) for 2, 4 and 8 hours. Left and right figures highlight the data from macaibo and pitiúba, respectively. This data refers to the relative ^{13}C-enrichment ($R^{13}C$) normalized according to the ^{13}C-enrichment in glucose from each genotype. Values with different letters are significantly different, as indicated by analysis of variance (ANOVA) and the test of Tukey ($P < 0.05$).

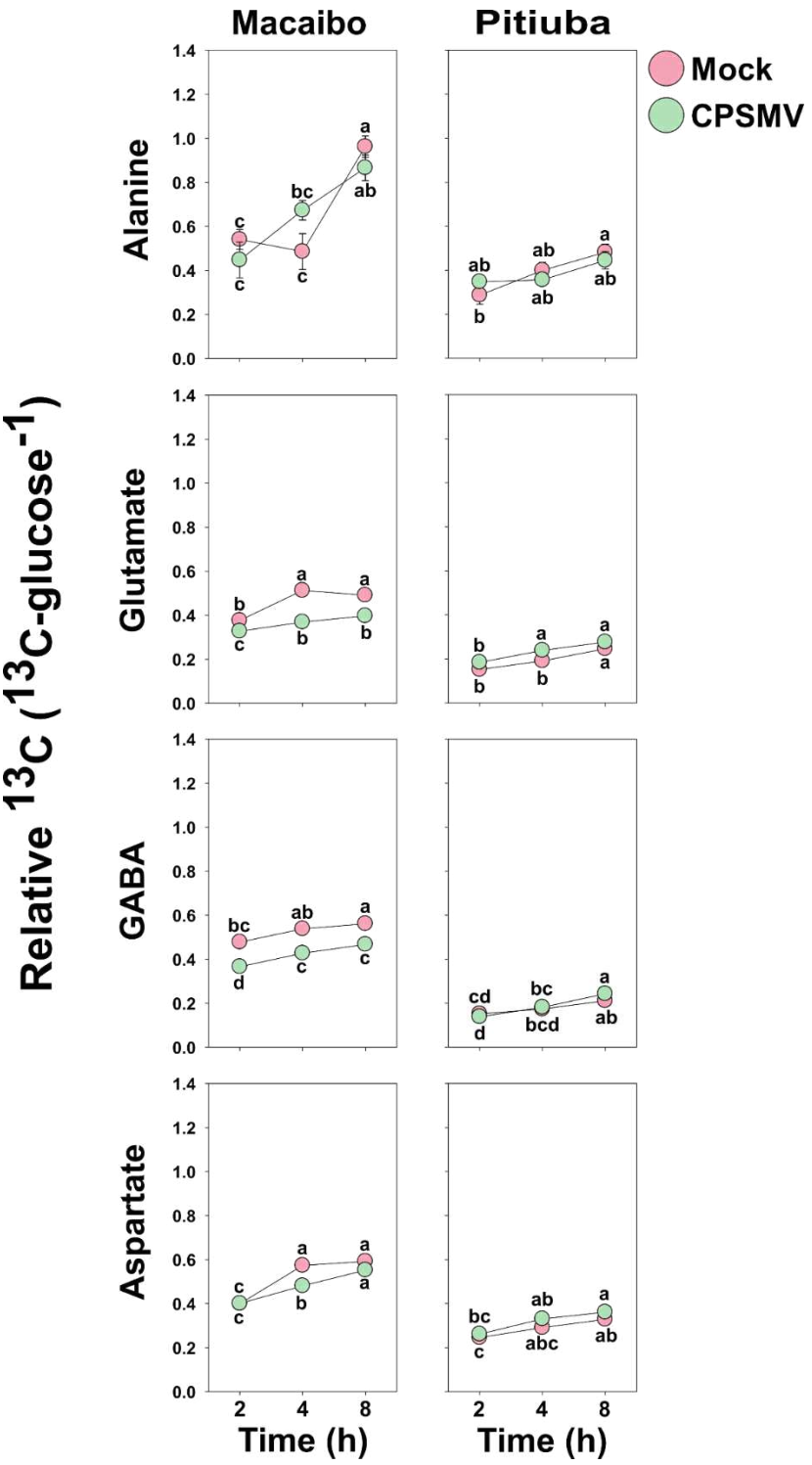

Figure S4. ^{13}C-enrichment in alanine, glutamate, GABA and aspartate from local and systemic macaibo and pitiúba leaves inoculated with mock or cowpea severe mosaic virus (CPSMV) for 2, 4 and 8 hours. Left and right figures highlight the data from macaibo and pitiúba, respectively. This data refers to the relative ^{13}C-enrichment ($R^{13}C$) normalized according to the ^{13}C-enrichment in glucose from each genotype. Values with different letters are significantly different, as indicated by analysis of variance (ANOVA) and the test of Tukey ($P < 0.05$).

Table S1. List of metabolites that have significant ^{13}C-enrichment in leaves inoculated with mock or CPSMV (cowpea severe mosaic virus). The relative ^{13}C-enrichment ($R^{13}C$) in the metabolites was analysed by analysis of variance (ANOVA) using the entire data set, which refers to all time points and treatments from both genotypes.

Metabolites	**Macaibo and Pitiúba**
Alanine	$P < 0.05$
Aspartate	$P < 0.05$
Citrate	$P < 0.05$
Fumarate	$P < 0.05$
Fructose	$P < 0.05$
GABA	$P < 0.05$
Glucose	$P < 0.05$
Glutamate	$P < 0.05$
Glycerate	$P < 0.05$
Glycine	$P < 0.05$
Glycolate	ns
Isoleucine	$P < 0.05$
Lactate	ns
Leucine	ns
Malate	$P < 0.05$
myo-inositol	$P < 0.05$
2-Oxoglutarate	$P < 0.05$
Pyruvate	$P < 0.05$
Serine	$P < 0.05$
Shikimate	$P < 0.05$
Succinate	$P < 0.05$
Sucrose	$P < 0.05$

Table S2. Comparison of the relative ^{13}C-enrichment ($R^{13}C$) between macaibo and pitiúba leaves infected with CPSMV (cowpea severe mosaic virus) in each time point. This analysis was carried out by Student's *t* test ($P < 0.05$) using the raw data (non-normalized by glucose) of macaibo and pitiúba leaves infected with CPSMV. HAI – Hours after CPSMV inoculation.

	Macaibo *vs* Pitiúba (raw $R^{13}C$ data)		
Metabolites	**2 HAI**	**4 HAI**	**8 HAI**
Alanine	$P < 0.05$	ns	ns
Aspartate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Citrate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Fumarate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Fructose	$P < 0.05$	$P < 0.05$	$P < 0.05$
GABA	ns	ns	ns
Glucose	$P < 0.05$	$P < 0.05$	$P < 0.05$
Glutamate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Glycerate	$P < 0.05$	ns	ns
Glycine	$P < 0.05$	$P < 0.05$	$P < 0.05$
Isoleucine	ns	ns	$P < 0.05$
Malate	$P < 0.05$	$P < 0.05$	$P < 0.05$
myo-inositol	$P < 0.05$	$P < 0.05$	$P < 0.05$
2-Oxoglutarate	ns	ns	$P < 0.05$
Pyruvate	ns	ns	ns
Serine	ns	$P < 0.05$	ns
Shikimate	$P < 0.05$	ns	$P < 0.05$
Succinate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Sucrose	$P < 0.05$	$P < 0.05$	$P < 0.05$

Table S3. Comparison of the relative ^{13}C-enrichment ($R^{13}C$) between macaibo and pitiúba leaves infected with CPSMV (cowpea severe mosaic virus) in each time point. This analysis was carried out by Student's *t* test ($P < 0.05$) using the $R^{13}C$ data normalized by the ^{13}C-enrichment in glucose of macaibo and pitiúba leaves infected with CPSMV. HAI – Hours after CPSMV inoculation.

	Macaibo *vs* Pitiúba ($R^{13}C$ normalized by ^{13}C-glucose)		
Metabolites	**2 HAI**	**4 HAI**	**8 HAI**
Alanine	ns	$P < 0.05$	$P < 0.05$
Aspartate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Citrate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Fumarate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Fructose	$P < 0.05$	ns	$P < 0.05$
GABA	$P < 0.05$	$P < 0.05$	$P < 0.05$
Glucose	$P < 0.05$	$P < 0.05$	$P < 0.05$
Glutamate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Glycerate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Glycine	$P < 0.05$	$P < 0.05$	$P < 0.05$
Isoleucine	ns	ns	$P < 0.05$
Malate	$P < 0.05$	$P < 0.05$	$P < 0.05$
myo-inositol	$P < 0.05$	$P < 0.05$	$P < 0.05$
2-Oxoglutarate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Pyruvate	ns	ns	$P < 0.05$
Serine	$P < 0.05$	$P < 0.05$	ns
Shikimate	$P < 0.05$	$P < 0.05$	$P < 0.05$
Succinate	$P < 0.05$	$P < 0.05$	ns
Sucrose	$P < 0.05$	$P < 0.05$	$P < 0.05$

4 CONCLUSION

Beyond providing unprecedented information concerning the metabolic responses of cowpea plants to CPSMV infection, our study unveiled that the dynamic of accumulation/degradation of primary metabolites is altered in the CPSMV-resistant macaibo genotype upon CPSMV infection. However, the topology of the primary metabolic network and the level of secondary metabolites remained relatively stable upon CPSMV infection, highlighting that the changes at primary metabolism level were sufficient to struggle CPSMV infection. We further showed that the primary metabolism of the CPSMV-resistant macaibo genotype is substantially different from the CPSMV-susceptible pitiúba genotype. Among the metabolic differences between these genotypes, we consistently observed that the accumulation of shikimate is different between macaibo and pitiúba, in which macaibo showed higher glycolytic fluxes toward this metabolite, despite the presence/absence of CPMSV infection. Given the well-established role of shikimate for plant defence, this result suggests that macaibo may have a constitutive higher flux toward the secondary metabolism. However, our results highlighted that CPSMV infection marginally affected the level of secondary metabolites. Thus, although shikimate and other secondary metabolites downstream in the shikimate pathway may have a role for macaibo CPSMV resistance, only a LC-MS characterization of pitiúba leaves infected with CPSMV alongside with macaibo will unveil whether this pathway is important for CPSMV resistance. Therefore, further genetic and LC-MS characterization of the genotypes used here is needed to fully understand the metabolic basis of the cowpea CPSMV resistance.

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APPENDIX A – LIST OF FIGURES

CHAPTER I

CHAPTER II

APPENDIX B – LIST OF TABLES