1 ***Cassava common mosaic virus* causes photosynthetic alterations associated with**
2 **changes in chloroplast ultrastructure and carbohydrate metabolism of cassava**
3 **plants**

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26 **Running title:** CsCMV infection induced chloroplast and metabolic alterations

27

28 ***Main conclusion***

29 CsCMV infection adversely modified chloroplast structure and had functional effects on
 30 chloroplasts during the course of viral infection, associated with metabolic adjustment
 31 in cassava source leaves, which would partly explain cassava root yield losses.

32

33 Abstract

34

35 *Cassava common mosaic virus* (CsCMV) is a potexvirus that causes systemic
 36 infections in cassava plants, leading to chlorotic mosaic and producing significant yield
 37 losses. To date, the physiological alterations and the mechanism underlying biotic stress
 38 during the cassava-CsCMV compatible interaction remains unknown. In this study, we
 39 found that CsCMV infection adversely modified chloroplast structure and had
 40 functional effects on chloroplasts in source leaves during the course of viral infection.
 41 Extrusion of the chloroplast membrane with amoeboid-shaped appearance was observed
 42 in infected mesophyll cells. These alterations were associated with lower relative
 43 chlorophyll content, and reduced PSII efficiency and CO₂ fixation. Moreover, an
 44 oxidative stress process was observed in CsCMV-infected plants. Strong declines in the
 45 maximum quantum yield of primary photochemistry (F_v/F_m) were observed in infected
 46 plants. Furthermore, the analysis of Chlorophyll-a fluorescence (ChlF) evidenced a
 47 progressive loss of both oxygen evolving complex activity and “connectivity” within
 48 the tripartite system (core antenna-LHCII-Reaction Centre). Other effects of the
 49 pathogen included reduction of starch and maltose content in source leaves, and a
 50 significant increase of the sucrose/starch ratio, which indicates alteration pattern of
 51 carbon. Our results suggest that CsCMV induces chloroplast distortion associated with
 52 progressive chloroplast function loss and diversion of carbon flux in source leaf tissue,
 53 which should be key in inducing yield losses of infected crops.

54

55 **Key words:** biotic stress, cassava, chloroplast alteration, CsCMV, OJIP test, potexvirus

56

57

58 **Abbreviations:**

59 AGPase, ADP-glucose pyrophosphorylase

60 ChlF, Chlorophyll-a fluorescence

61 CsCMV, *Cassava common mosaic virus*

62 DAP, Days After Planting

63 FW, Fresh Weight

64 OEC, Oxygen Evolving Complex

65 PSII, Photosystem II

66 RC, Reaction Center

67 ROS, Reactive Oxygen Species

68

69 Introduction

70 Cassava (*Manihot esculenta* Crantz) is one of the most widely grown tuber crops
 71 and an important food security crop for smallholder farmers, especially in low-income,
 72 food-deficit areas (Bellotti et al. 1999). Its storage roots are the main organ, which is
 73 marketed as feed resource and feedstock for starch extraction, bio-energy production,
 74 and pharmaceutical and textile industries; accordingly, cassava is a widely recognized
 75 sustainable crop in the context of the global changing climate (FAO 2013; De Souza et
 76 al. 2017; Marx 2019). Therefore, its productivity is conditioned by the starch
 77 accumulation from CO₂ fixation and the source-sink relationship (Yan et al. 2019).
 78 Since cassava is vegetatively propagated, its yield is greatly restrained by viral diseases
 79 (Legg et al. 2015). Among all reported cassava-infecting potexviruses (Lozano et al.
 80 2017), *Cassava common mosaic virus* (CsCMV) has been identified as the only one
 81 able to cause disease in single infection and to produce significant yield losses (Costa
 82 and Kitajima 1972; Venturini et al. 2016; Zanini et al. 2020). This pathogen has been
 83 detected infecting cassava crops in South America (Calvert et al. 2012; Chaparro-
 84 Martinez and Trujillo-Pinto 2003; Silva et al. 2011; Fernandez et al. 2017; Zanini et al.
 85 2018) and recently in China (Tuo et al. 2020).

86 The typical symptoms of CsCMV infection, known as cassava common mosaic
 87 disease (CCMD), vary in severity and include a wide range of foliar chlorotic mosaic
 88 (light green chlorotic areas, interspersed with dark green areas), sparsely distributed in
 89 the leaf blade of cassava plants (Zanini et al. 2018). This kind of symptoms might be the
 90 consequence of chloroplast deformation and shut-off induced by the virus infection (Li
 91 et al. 2016a). The viral influence on chloroplast structure and function, which usually
 92 leads to photosynthetic impairment, is common across diverse plant-virus interactions
 93 (Zhao et al. 2016). Accordingly, viruses interfere with photochemical reactions by

reducing the abundance of key proteins involved in the photosynthetic electron transport chain (Souza et al. 2019; and references therein). In this context, alterations in electron transport might generate reactive oxygen species (ROS) (Rodríguez et al. 2010). The ROS-antioxidant balance determines, to a large extent, the cellular redox state, which plays a central role in environmental stress perception and defense response modulation (Foyer and Noctor, 2005).

To enable their multiplication, plant viruses have the ability to rearrange host metabolism and cellular machinery (Souza and Carvalho 2019). A wide range of physiological and biochemical disorders are triggered from cell infection, including relocation of photoassimilates, redox imbalance and premature senescence, with significant economic losses (Rodríguez et al. 2010, 2012; Souza and Carvalho 2019). Viral infections lead to a decline in CO₂ fixation, which could be directly related to a decrease in carbohydrate accumulation, reducing plant growth and development (Sade et al. 2013; Nuwamanya et al. 2017). On the contrary, other studies have demonstrated that soluble sugars and starch accumulate in the infected leaves, where photosynthesis is reduced (Rodriguez et al. 2010; Andreola et al. 2019; De Haro et al. 2019). Furthermore, abnormal accumulation or depletion of starch in systemically infected tissues was reported in *Cucumber mosaic virus* (CMV, Cucumovirus)-marrow interaction (Técsi et al. 1996). Therefore, the mechanisms associated with changes in carbohydrate metabolism are specific to the plant-virus pathosystem; to our knowledge, the effect of CsCMV infection on sugar and starch content has still not been studied.

Given the significant yield losses caused by CsCMV (from 30% to 60%) (Costa and Kitajima 1972; Venturini et al. 2016; Zanini et al. 2020), it is imperative to investigate the physiological basis underlying cassava-CsCMV interplay. To extend the knowledge acquired in previous studies by Tascon et al. (1975), we focused our

research on morphological and functional alteration of cassava chloroplast. Taking into account the theoretical background mentioned above, we hypothesized that CsCMV would induce anomalies in the chloroplasts associated with reduced photosystem II (PSII) electron transport activity and redox imbalance. We further proposed that since CO₂ fixation decreases, sugar and starch content will be negatively affected. Therefore, the present study provides, for the first time, information about chloroplast structure and progression of the CCMD on chloroplast function in the compatible cassava-CsCMV interaction.

127

128 **Materials and Methods**

129

130 **Plant materials**

131 Twenty *in-vitro* grown, healthy cassava v. ICA Negrita (CM3306-4) plants were
132 used. Sixty days after acclimation, 10 plants were graft-inoculated with budwood from
133 CsCMV-affected cassava plants and the other 10 were autografted to maintain their
134 healthy condition. Yellowing and blotchy mosaic symptoms were visible 20 days after
135 inoculation. These plants were transplanted in the field for macro-propagation and as a
136 source of stem cuttings for subsequent greenhouse trials.

137

138 **Experimental setup**

139 Cassava plants were propagated clonally from segments (approximately 20 cm
140 in length) of parental stems with at least 6 nodes. The plants were grown individually in
141 plastic pots (3 L) containing a 3:1 mixture (v/v) of potting soil and sand mixture, in a
142 naturally illuminated greenhouse. All experiments were carried out in summer (average
143 temperature 28 ± 5 °C). Thirty cassava stakes were used for each sanitary condition:

144 non-infected plants (control) and CsCMV-infected ones, arranged in three completely
145 randomized blocks.

146 Measurements of number of nodes and number of leaves per bud, SPAD and
147 chlorophyll fluorescence were taken in three ontological times: 60, 75 and 90 days after
148 planting (DAP) of cassava plants, encompassing the key stages of cassava growth, from
149 the 'seedling' stage (young cassava plants that start from stem cuttings, until 60 DAP)
150 to the formation of root system (until 110 DAP). Height was measured at 60, 75, 90 and
151 210 DAP (cassava growth stage of starch maturity) and photosynthetic rate was
152 estimated at 90 and 210 DAP.

153 Leaf tissue samples (excluding petioles) were taken from the fourth leaf
154 (counting from the base to the top) of each plant at 90 DAP. They were immediately
155 frozen in liquid nitrogen and stored at -80 °C for subsequent biochemistry
156 determinations. These leaves represent a source organ.

157

158 **Serological virus detection**

159 CsCMV was detected by double antibody sandwich enzyme-linked
160 immunosorbent assay (DAS-ELISA) performed in polystyrene microtitre plates with
161 anti-CsCMV-IgG as described Zanini et al. (2018). All plants were tested.

162

163 **Transmission electron microscopy - Immunogold labelling**

164 Small tissue sections (0.2 cm wide × 0.5 cm long) sliced from the middle part of
165 CsCMV-infected leaves were fixed in 2% paraformaldehyde and 2% glutaraldehyde in
166 0.01 M phosphate buffer, pH 7.2, for 1 day. Then, they were postfixed in 1% OsO₄, in
167 the same buffer and embedded in LRGold, as previously described (Kitajima 1997,

168 Maunsbach 1998). Sections were cut with a diamond knife using an ultra-microtome
169 and collected onto nickel grids. For immuno-electron microscopy, sections mounted on
170 nickel grids were preincubated with 1% bovine serum albumin (BSA) in 1% phosphate
171 buffered saline (PBS) overnight, and then treated with specific antiserum anti-CsCMV
172 (Zanini et al. 2018) for six hours. The sections were then exposed to gold conjugated
173 protein A of 5 nm in diameter (Zymed, now part of the Invitrogen). Sections from
174 uninfected cassava leaves were used as additional controls in the immunolabelling
175 experiments. They were examined under a JEOL JEM-1200 transmission electron
176 microscope (TEM, JEM 1200 EXII, Jeol, Tokyo, Japan).

177

178 **Chlorophyll content, chlorophyll-a fluorescence and gas-exchange measurements**

179 **Chlorophyll content.** The relative chlorophyll (Chl) content was determined
180 with a portable meter (Hansatech, Chlorophyll Content Meter), using dual wavelength
181 optical absorbance measurements (620 and 940 nm). The measurements were taken
182 simultaneously with Chlorophyll-a fluorescence (ChlF), as described below.

183 **Chlorophyll-a fluorescence transient.** ChlF emission was measured with a
184 Pocket-PEA fluorometer (Plant Efficiency Analyzer, Hansatech Instruments Ltd.,
185 King's Lynn, Norfolk, UK). The polyphasic OJIP fluorescence kinetics was used to
186 evaluate the PSII activity in CsCMV-infected and non-infected cassava plants. Before
187 ChlF measurement was taken, the fourth developed leaves were dark-adapted using leaf
188 clips for at least 30 min in order to allow full oxidation of the reaction centers (RC); the
189 minimum fluorescence or F_0 or O step was obtained at $\sim 50 \mu\text{s}$. Then, an actinic 1-
190 second light pulse of $3500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ was applied to reach the maximum
191 fluorescence emission (F_m or P step), at about 300 ms. The intermediate steps, called J

192 and I, were recorded at 2 ms and 30 ms, respectively. The OJIP parameters were
193 calculated using the Pocket-PEA manufacture software, following Strasser et al. (2004).
194 The parameters are described in Supplementary Table S1.

195 Changes in the oxygen evolving complex (OEC) activity and in the connectivity
196 at PSII were calculated according to Chen et al. (2016). To reveal changes in OEC (K-
197 band), fluorescence curves were normalized at both the O and J steps, rendering W_{OJ}
198 curves. Next, W_{OJ} curves from control plants were subtracted from treated ones
199 ($W_{OJ(Treated)} - W_{OJ(Control)}$) to disclose the K bands (ΔW_{OJ}). Positive values in these bands
200 denote damage to the OEC. To detect L bands, curves were normalized between 0 and
201 300 μs , and plotted as W_{OK} curves. The subtraction between $W_{OK(Treated)} - W_{OK(Control)}$
202 was plotted to obtain ΔW_{OJ} curves, which allowed us to visualize L bands. The positive
203 values of ΔW_{OJ} curves are proportional to the loss of connectivity at PSII.

204 **Net photosynthesis rates.** Net CO_2 assimilation rates were measured in the
205 fourth fully expanded leaves of cassava plants at 90 and 210 DAP with a portable
206 photosynthesis system LI-6400 XT equipped with a LED leaf cuvette (Li-Cor, Lincoln,
207 NE, USA). The concentration of CO_2 inside the chamber was kept at 400 $\mu mol CO_2$
208 mol^{-1} . The light source was set at saturating incident photosynthetic photon flux density
209 (PPFD) of 1500 $\mu mol m^{-2}s^{-1}$ (90% of red and 10% of blue light) and the temperature
210 was maintained at 25 °C.

211

212 **Determination of the effect of CsCMV on cassava carbohydrate**

213 **Ethanol leaf extract.** Frozen tissue (75 mg) was ground with liquid nitrogen
214 and cold homogenized with 750 μL of 80% ethanol. The extract was poured into a
215 previously cooled microtube and centrifuged at 12,000 g at 4 °C for 10 min. The

216 samples were kept on ice and aliquots were separated to measure the total antioxidant
217 capacity using FRAP (*Ferric Reducing Ability of Plasma*). The remaining extract was
218 resuspended in vortex and taken to a thermal bath at 80 °C for 20 min. The samples
219 were then centrifuged at 12,000 g at 4 °C for 10 min and aliquots were separated from
220 the supernatant to measure total sugars. The insoluble fraction was used for
221 quantification of starch content.

222 **Total soluble sugars.** Quantification of total soluble sugars was adjusted to a
223 final reaction of 200 µL in polystyrene plates. Ethanolic leaf extract and Anthrone
224 reagent (1 µL each) were placed in each well (Fales 1951). The blank contained H₂O
225 and Anthrone reagent. The mixture was incubated at 4 °C for 10 min, then at 80 °C for
226 30 min, and subsequently allowed to cool 20 min at room temperature. Glucose was
227 used as standard to measure the total soluble sugars content, expressed as mg of glucose
228 per g of FW.

229 **Sugar determination (HPLC).** Frozen leaf samples (0.1 g FW) were ground to
230 a fine powder with liquid nitrogen, homogenized and suspended in 0.9 mL hot 80%
231 ethanol, and kept at 80 °C for 2 h. Then, 0.9 mL dH₂O was added to each sample;
232 samples were incubated at 99 °C for 10 min and centrifuged at 12,000 g for 5 min. The
233 supernatant was filtered using a nitrocellulose filtration membrane (0.22 µm pore size).
234 Sucrose, glucose, fructose and maltose were determined by high-performance liquid
235 chromatography (HPLC) (Shimadzu) using an amine column, isocratic acetonitrile:
236 water (81:19) flow (1 mL/min), at 30 °C. Sugars were identified by their retention times
237 and quantified according to standards.

238 **Starch content.** Starch content was determined in the pellet, from reducing
239 sugars released after hydrolysis with the enzyme α -amylglucosidase (Sumner and

240 Somers 1944), using glucose as a standard. Starch content in the source organs was
241 expressed as mg per g of FW of plant material.

242 **AGPase activity.** The assay was performed in the reverse direction using
243 phosphoglucosyltransferase and Glucose-6-phosphate dehydrogenase to couple Glucose-1-
244 phosphate formation in real time to NADP⁺ reduction and then measured by
245 spectrophotometers. The crude extracts were obtained as described by Li et al. (2016b),
246 adapted to cassava leaves. Briefly, 0.1 g of fresh tissue fully homogenized on ice in a
247 0.3 ml pre-cooled solution composed of 100 mM Hepes-NaOH at pH 7.4, 8 mM MgCl₂,
248 2 mM EDTA, 12.5% (v/v) glycerol, 5% (v/v) polyvinylpyrrolidone, and 50 mM β -
249 mercaptoethanol. Then, a 20 μL aliquot of the crude enzyme extract was used to start
250 the assay as detail Li et al. (2016b). Absorbance values at 340 nm of the reaction
251 solution were normalized by absorbance values at 340 nm resulting from the control.
252 The AGPase activity was then estimated with the normalized absorbance values and
253 was expressed in enzyme units per mg of protein, calculated according to Kulichikhin et
254 al. (2016). Protein content was determined in leaf extracts by Bradford (1976) assay.

255

256 **Determination of Total antioxidant capacity (FRAP)**

257 The FRAP assay was used to determine the total antioxidant capacity of the
258 samples (Benzie and Strain 1996); the protocol was adjusted for performance in a final
259 reaction of 200 μL in polystyrene plates. In each well, 2 μL of ethanolic extract solution
260 (see ethanolic leaf extract) were placed to react with FRAP reagent in the dark at room
261 temperature for 20 min and the absorbance at 600 nm was recorded. Trolox® (6-
262 hydroxy- 2,5,7,8- tetramethylchroman- 2-carboxylic acid, Sigma-Aldrich) solutions of
263 known concentration, within in the range of 12.5 and 87.5 μM, were used for

264 calibration. Total non-enzymatic antioxidant activity was expressed as μ moles per g of
265 FW.

266

267 **Statistical analysis**

268 Experiments were performed in a completely randomized design. Mixed linear
269 model was performed using InfoStat (Di Rienzo et al. 2019). Mean separation was
270 accomplished using Least Significant Differences (LSD) via Fisher test at the 95%
271 confidence level.

272

273

274 **Results**

275

276 *Chlorotic symptom during CsCMV infection: effect on chloroplast ultrastructure*

277 Cassava plants infected with CsCMV exhibited the typical symptoms of CCMD,
278 such as foliar chlorotic mosaic and mild leaf distortion (Fig. 1). The mosaic symptom
279 was evident since leaf appearance. Furthermore, those plants that were self-grafted
280 remained asymptomatic (non-infected plants). Systemic spreading of virus in plant
281 tissues was confirmed by DAS ELISA (data not shown). Even though viral infection
282 showed conspicuous foliar mosaic, no differences in the fully expanded leaf area were
283 observed between healthy and CsCMV-infected plants (Supplementary Fig. S1). Yet,
284 growth analysis showed that viral infection significantly affected ($p < 0.05$) the absolute
285 and relative elongation rate (AER and RER, respectively). Indeed, the analysis of AER
286 of infected plants showed a rapid stimulation of plant height, with a subsequent
287 decrease of the rate over the days with respect to non-infected plants (Supplementary
288 Fig. S2). The number of nodes and leaves per node increased over time; however, no
289 significant differences were observed between plants of different sanitary conditions
290 (Supplementary Fig. S3).

291 The structure of chloroplasts revealed abnormalities in mesophyll CsCMV-
292 infected cells, which included dramatic chloroplast malformations, such as small
293 vesicles or vacuoles in stroma, disorganized grana stacks and irregular arrangement of
294 stroma lamellae (Fig. 2). Furthermore, CsCMV, as a potexvirus member, have flexuous
295 filamentous particles of 470–580 nm in length, which were observed accommodated in
296 inclusion bodies of fibrous masses containing these filamentous particles, running more
297 or less parallel to each other, but not in a definite arrangement, in the cellular cytoplasm
298 near the chloroplast membrane (Fig. 2).

299

300 *Photosynthetic activity is compromised in CsCMV- infected leaves*

301 Fast ChlF kinetics was measured to reflect the decrease of PSII activity induced
 302 by CsCMV in cassava plants. The value of the OJIP parameters, affected by the
 303 CsCMV, were normalized to the control ones and plotted in a radar graph to deploy a
 304 global view of the structure and function of PSII electron transport (Fig. 3b).
 305 Accordingly, the maximum quantum efficiency F_v/F_m was negatively affected as the
 306 disease progressed, becoming more evident after 90 DAP. Such changes occurred due to
 307 the reduction of both minimum (F_o) and maximum (F_m) ChlF emission. The energy
 308 flow taking place at the reaction centers (RC) was also altered by the CsCMV infection.
 309 Such event begins at light absorption (ABS) by PSII antenna pigments and ends at
 310 reduction of the end electron acceptors at the photosystem I (PSI) electron acceptor side
 311 (RE) driven by PSI. As of 60 DAP, a continuous energy loss as latent heat (DI_o/RC)
 312 was observed. Then, changes in the size of the antenna complex (ABS_o/RC) and energy
 313 capture (TR_o/RC) took place after 75 DAP. PSI electron acceptor side (RE_o/RC) also
 314 increased during the same period. Absolute performance index (PI_{abs}) of infected plants
 315 slumped by 33% after 60 DAP with respect to non-infected plants. Likewise, total
 316 performance index (PI_{total}) diminished, but recovered slightly 90 DAP. Significant
 317 symptoms of chlorosis of cassava leaves (SPAD index; Fig. 3b) were evident as early as
 318 the 60 DAP. As a consequence of chloroplast damage and the energetic imbalance at the
 319 PSII electron transport, reducing power for photosynthesis was reduced. This result may
 320 explain the reduction of the photosynthetic rate observed 90 and 210 DAP (13.5% to
 321 24.2%, respectively) (Fig. 3a).

322 The differences of the normalized ChlF curves (ΔW_{OK} and ΔW_{OI}) revealed the
 323 appearance of hidden steps, such as the K and L bands (OLKJIP). Positive bands (K

band) occurring at about 300 μ s are considered to denote failure at the OEC during 60, 75, and 90 DAP in CsCMV-infected plants (inset Fig. 3c; Oukarroum et al 2007). Moreover, the fluorescence rise during the first 150 μ s (L-band) is attributed to the loss of energetic connectivity between PSII units (Ripoll et al 2016). Here, the appearance of L band increased from 60 to 90 DAP (inset Fig. 3d).

CsCMV infection induce redox imbalance in cassava plants

The FRAP assay offers a putative index of antioxidant or reducing state. An indirect estimation of the total non-enzymatic antioxidant content of the extract showed significant differences between the means of CsCMV-infected plants and the control plants (Fig. 4). The reduction of the antioxidant capacity was about 20.8% in virus-infected plants.

CsCMV infection: effects on carbohydrate and starch accumulation

Starch content in infected cassava leaves decreased significantly (by 22.8%), with respect to control (Fig. 5a). Total soluble sugar content (Fig. 5b) and AGPase activity were not affected in infected leaves (Fig. 5c).

A highly detailed profile of soluble sugars evidenced a decrease in maltose content without significant differences in fructose, glucose or sucrose in CsCMV-infected leaves with respect to control plants (Table 1). The sucrose/starch ratio showed a significant increase with respect to non-infected plants (Table 1).

346 **Discussion**

347 Cassava-CsCMV compatible viral infection causes 30-60% yield reduction of
348 cassava roots (Costa and Kitajima 1972; Venturini et al. 2016; Zanini et al. 2020).
349 These yield losses reflect the final result of a series of physiological events that occurred
350 at earlier stages of viral interaction. The present study was focused on chloroplast
351 morphological alteration and functional effects on source leaves during the course of
352 viral infection. The mosaic symptoms induced by CsCMV were associated with
353 structural changes of chloroplasts, decrease in relative Chl content, electron transport
354 rate alteration, photoinhibition, reduction of CO₂ fixation, reduction of starch, and
355 maltose accumulation associated with increase in sucrose/starch ratio and drop of total
356 antioxidant content.

357 The interaction between chloroplast and the invading virus plays a critical role in
358 viral infection and pathogenesis (Zhao et al. 2016). Our previous results showed
359 inclusion bodies of fibrous masses containing filamentous particles in the cellular
360 cytoplasm near the chloroplast membrane (Zanini et al. 2014). In the present study,
361 which was focused on chloroplast structure, we observed extrusion of the chloroplast
362 membrane with amoeboid-shaped appearance, among other results (Fig. 2b). Several
363 research works have shown that viruses can interact with chloroplast proteins and
364 induce the formation of membrane vesicles during viral replication, which impairs
365 chloroplast functions in plants (Liu et al. 2014; Li et al. 2016a; Zhao et al. 2016; Souza
366 and Carvalho 2019). For example, high levels of *Potato virus X* (PVX, *Potexvirus*)-Coat
367 Protein (CP) caused structural alteration of the chloroplast membranes, thylakoid grana
368 and invagination of the cytoplasm within these organelles; it was also found to interact
369 with the plastocyanin (Zhao et al. 2016).

370 The development of mosaic symptom induced by CsCMV not only was a
 371 consequence of the chloroplast structure alteration in cassava mesophyll cells (Fig. 2),
 372 but also severely inhibited its function (Fig. 3). Our results agree with findings of Liu et
 373 al. (2014), who reported that visible symptoms of chlorosis in cassava plants infected
 374 with *African cassava mosaic virus* (ACMV, *Begomovirus*) were associated with
 375 chlorophyll breakdown (reduction of chlorophyll content and fewer grana stacks) and
 376 upregulation of chlorophyll degradation transcript genes. For a long time, the
 377 chloroplast-virus interaction has been a matter of debate due to its complexity. We
 378 noticed that CsCMV infection negatively affected the CO₂ gas-exchange rate, with a
 379 constant fall over the progress of the disease (Fig. 3a). This phenomenon could be
 380 explained by the lower production of ATP and NADPH, due to the closure of RC, as
 381 indicated by the increment in ABS_o/RC values (Fig. 3b). On the other hand, virus
 382 infection could have reduced the carboxylation capacity through impairment of Rubisco
 383 activity (Sampol et al. 2003). According to the analysis of ChlF signals, CsCMV
 384 showed a severe impact on the photosynthetic light primary reaction at 90 DAP (Fig.
 385 3b).

386 The loss of PSII electron transport efficiency is regarded as a common
 387 phenomenon under biotic stress. As expected, the OJIP- test showed significant changes
 388 of most of the ChlF parameters over time in CsCMV infected plants in relation to the
 389 healthy ones. For instance, the viral infection decreased the photochemical efficiency of
 390 PSII (F_v/F_m ; Fig. 3b), whereas it increased light energy dissipation (DI_o/RC)
 391 significantly, and decreased performance index (PI_{abs}) and alteration in the linear
 392 electron transport (ET_o/RC) in PSII. According to Gallé and Flexas (2010), a decrease
 393 in F_v/F_m is due to an increment in thermal dissipation (non-photochemical quenching) at
 394 the expense of photochemical activity. Furthermore, when this parameter becomes

markedly reduced, it is considered as an indication of photoinhibition or PSII photo-damage (Maxwell and Johnson, 2000), as is the case of CsCMV infected-leaves at 90 DAP. Similar results were found in *Sunflower chlorotic mottle virus* (SuCMoV, *Potyvirus*), with degradation of D1 protein, an essential protein of PSII functionality, resulting in photoinhibition/photodamage, and drop of F_v/F_m parameter (Rodríguez et al. 2010).

Oxygen evolving complex (OEC), a Mn_4O_5Ca protein cluster embedded in the PSII, is essential in the photosynthesis process. It has been demonstrated that the viral CP, or some other products synthesized as a result of the viral infection, decreases the amount of OEC polypeptides, as reported in tobacco leaves infected with *Cucumber mosaic virus* strain Y (CMV(Y), *Cucumovirus*) (Takahashi and Ehara 1992). In our work, a progressive loss of OEC activity was revealed by the K-band starting 60 DAP in CsCMV-infected leaves (inset Fig 3c). Under particular stressful conditions, the K-band was demonstrated to indicate a disruption of the OEC (Kalaji et al., 2016). Likewise, the OJIP analysis evidenced that the slope between F_0 and F_{150} (L-band) was sensitive to the CsCMV attack, which was progressively increased up to 90 DAP (inset Fig. 3d). This phenomenon occurs due to a loss of “connectivity” within the tripartite system (core antenna-LHCII-Reaction Centre) under particular abiotic stress conditions (Strasser et al. 2004; Kalaji et al., 2016); here it is reported for virus infection for first time.

The virus ability to impair chloroplast function and disrupt the photosynthetic electron transport chain ultimately leads not only to the decrease of the carboxylation activity but also to ROS increase (Rodríguez et al. 2010; 2012; Souza et al. 2019). CsCMV induced redox alteration demonstrated by the lower antioxidant capacity (FRAP), which might indicate an oxidative stress process. Furthermore, the CO_2

420 fixation decrease might be directly related to soluble sugar production. Nuwamanya et
 421 al. (2017) demonstrated that cassava brown streak disease (CBSD), caused by
 422 potyviruses, leads to a general reduction in primary carbohydrate composition (total
 423 reducing sugar and starch contents) in leaves; these effects correlated with the negative
 424 effect on photosynthetic apparatus of infected leaves. However, our results did not show
 425 such significant differences in the total soluble sugar contents in CsCMV- infected
 426 source leaves with respect to non-infected plants. The differences between results might
 427 be related to the specific source tissue analyzed during our work. These results led us to
 428 ask about the origin of these sugars, since photosynthetic activity was decreased by viral
 429 infection. One possible answer might be related to the findings of Sharkey et al. (1985)
 430 that, at low rates of photosynthesis, carbon would preferably be used for sucrose
 431 synthesis rather than for storage in starch form. Accordingly, present study showed a
 432 rise of the ratio of carbon partitioned into sucrose versus starch (suc/starch ratio, Table
 433 1) in CsCMV-infected plants. Another possible answer may be starch breakdown,
 434 which provides a source of reduced carbon when photosynthesis cannot occur. Indeed,
 435 starch content was decreased during viral infection (Fig. 5a). However, maltose, which
 436 might be increased in leaves when starch catabolism is induced, showed a significant
 437 decrease in CsCMV- infected leaves (Table 1). In this context, we cannot rule out the
 438 fast maltose metabolism to viral replication. Moreover, the different sugars might be
 439 finely sensed and might provide a mechanism to conduct carbon flux during viral
 440 infection (Sade et al., 2013; Hulsmans et al. 2016). Besides metabolic flux diversion,
 441 another possible source of soluble sugar could be related to alteration of sugar
 442 transporters, which convert these `source` leaves into `sink` tissues (Kanwar and Jha
 443 2019), or a gluconeogenesis process induction. These possible sugar sources are not

444 mutually exclusive but might be operating in combination, and have to be explored in
445 future studies.

446 To conclude, cassava produces storage roots, which are the major sink for
447 storing starch derived from carbohydrate partitioning and long-distance sugar transport
448 from the leaves (Yan et al. 2019). The early alterations in photosynthesis and
449 chloroplast structure observed during the present work might be related to negative
450 impact on yield previously observed at a later stage (Zanini et al., 2020). Such changes
451 imply smaller reserve roots, i.e., fewer accumulated carbohydrates. Therefore,
452 understanding the metabolic alteration induced by CsCMV in source leaves at this early
453 stages is critical for improving cassava yield. This is the first report demonstrating
454 physiological alterations associated with chloroplast ultrastructure and function in the
455 cassava-CsCMV pathosystem.

456

457 **Disclosure of Potential Conflicts of Interest**

458 No potential conflicts of interest are disclosed.

459

460 **Author Contribution**

461 MR and AAZ designed the experiments. AAZ conducted the experiments. AAZ and
462 MR took the measurements. AC carried out in vitro culture and acclimatization of
463 cassava seedlings. LDF contributed with cassava plants and virus detection. DFL
464 contributed in the fluorescence analysis. AAZ and PP performed the statistical analysis
465 of data. AAZ and MR drafted and co-wrote the manuscript. AAZ, LDF, DFL and MR
466 contributed with the discussion and editing of the manuscript. MR conceived and
467 supervised the project.

468

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475 is fellow and MR is researcher of CONICET (Consejo Nacional de Investigaciones
476 Científicas y Técnicas, Argentina). MR and LDF are researchers of INTA.

477

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638

639

640 **Legends to figures and Table**

641

642 **Fig. 1** Plant material. (a, b) Healthy cassava plants (control). (c) Argentine field-
643 collected cassava plant showing cassava common mosaic disease symptoms. (d, e)
644 CsCMV-infected cassava plants 90 days after planting (DAP), showing mosaic leaf
645 symptoms with alternating green and yellow patches. Representative plants were used
646 for pictures.

647

648 **Fig. 2** Ultrastructure of chloroplasts from CsCMV-infected mesophyll cassava cells.
649 Transmission electron micrographs of a small sample (0.2 cm wide $\times$ 0.5 cm long) slice
650 from the middle part of leaf tissues from CsCMV- infected cassava plants. Leaf samples
651 were prepared for TEM and the capsid protein immunogold labeling of CsCMV was
652 performed as described in material and methods. (a, c) Capsid protein gold particle
653 deposits (white head arrows) were located in cytoplasm around chloroplasts (Chl),
654 where virus particles (VP) were present. CW- cell wall, Cyt- cytoplasm. (a, b) Black
655 arrows indicated chloroplast malformations, such as small vesicles or vacuoles in
656 stroma. Bars = 500 nm (a, b) and 200 nm (c)

657

658 **Fig. 3** PSII activity and CO₂ fixation in CsCMV- infected leaves. (a) Photosynthetic
659 rates of non-infected (control) and CsCMV- infected cassava plants at two growth
660 stages, 90 and 210 days after planting (DAP). (b) Radar plot showing OJIP test
661 parameters in cassava control plants and CsCMV- infected. Measurements were carried
662 out at 60 (blue), 75 (red) and 90 (black) DAP. Parameter abbreviations are described in
663 Supplementary Table S1. (c) Changes in the oxygen evolving complex (OEC, K band)
664 and (d) energetic connectivity of PSII units (L band) in non-infected (empty icons) and

665 CsCMV- infected cassava plants (black circles). (c, inset panel) WOJ: Normalized
 666 fluorescence kinetics from O (50 μ s) to J step (2 ms). Δ WOJ = $WOJ_{(Treated)} - WOJ_{(Control)}$,
 667 kinetics difference between treated and control plants, revealing the K band. (d, inset
 668 panel) WOK: Normalized fluorescence kinetics from O (50 μ s) to K step (300 μ s).
 669 Δ WOK = $WOK_{(Treated)} - WOK_{(Control)}$; kinetics difference between treated and control
 670 plants, revealing the L-band at 60 (blue), 75 (red) and 90 (black) DAP

671

672 **Fig. 4** Antioxidant system in CsCMV-infected leaves. FRAP was measured in cassava
 673 leaves at 90 DAP. Means of non-infected (white bars) and CsCMV- infected (black
 674 bars) plants. FW: fresh weight * statistically significant according to ANOVA followed
 675 by LSD Fisher test ($p < 0.05$) ($n = 30$)

676

677 **Fig. 5** Carbon metabolism on cassava-CsCMV interaction at 90 DAP. (a) Starch and (b)
 678 total soluble sugar content ($n = 30$ for each sanitary condition). (c) AGPase activity of
 679 cassava leaves of two sanitary conditions ($n = 40$). FW: fresh weight; pr: protein *
 680 statistically significant according to ANOVA followed by LSD Fisher test ($p < 0.05$)

681

682 **Table 1. Sugar profile ($mg\ g^{-1}$ FW) on CsCMV-infected leaves at 90 DAP.**

683 Abbreviations: Fru: fructose, Glc: glucose, Suc: sucrose, Malt: maltose. Results are
 684 means of sugar contents obtained by HPLC of 20 plants of each sanitary condition $\pm$ s.e.

685 ** statistically significant according to ANOVA followed by LSD Fisher test ($p < 0.05$)

686 * statistically significant according to ANOVA followed by LSD Fisher test ($p < 0.1$).

687

1 **Table 1. Sugar profile (mg g⁻¹ FW) on CsCMV- infected leaves at 90 DAP**

2

Source leaves	Fru	Glc	Suc	Malt *	Suc/Starch**
Non-infected (Control)	0.87 ±	2.29 ±	17.99 ±	4.73 ±	0.58 ± 0.11
	0.16	0.15	1.1	0.39	
CsCMV- infected	1.02 ±	2.28 ±	18.02 ±	3.95 ±	0.77 ± 0.11
	0.16	0.12	0.78	0.22	

3

4 Abbreviations: Fru: fructose, Glc: glucose, Suc: sucrose, Malt: maltose. Results are

5 means of sugar contents obtained by HPLC of 20 plants of each sanitary condition

6 ± s.e. ** statistically significant according to ANOVA followed by LSD Fisher test

7 (p < 0.05) * statistically significant according to ANOVA followed by LSD Fisher

8 test (p < 0.1)

9

10