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# Are Sunflower chlorotic mottle virus infection symptoms modulated by early increases in leaf sugar concentration?

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ABSTRACT

Symptom development in a susceptible sunflower line inoculated with Sunflower chlorotic mottle virus (SuCMoV) was followed in the second pair of leaves at different post-inoculation times: before symptom expression (BS), at early (ES) and late (LS) symptom expression. Sugar and starch increases and photoinhibition were observed as early effects BS, and were maintained or enhanced later on, however, chlorophyll loss was detected only at LS. Photoinhibition correlated with a drastic decrease in D1 protein level. The progress of infection was accompanied by decreasing levels of apoplastic reactive oxygen species (ROS). In infected leaves, higher antioxidant enzyme activities (superoxide dismutase, SOD; ascorbate peroxidase, APX; glutathione reductase, GR) were observed from BS. The purpose of this work was to evaluate whether the early increases in carbohydrate accumulation may participate in SuCMoV symptom expression. Similar effects on photoinhibition, apoplastic ROS generation and antioxidant activity were generated when healthy leaves were treated with sugars. These results suggest that photoinhibitory processes and lower apoplastic superoxide levels induced by SuCMoV infection may be modulated by sugar increases.



## Introduction

Sunflower chlorotic mottle virus (SuCMoV) is a potyvirus that causes systemic infections in sunflower plants, leading to chlorotic mottling and severe growth reductions and yield losses (Lenardon et al., 2001). In the sunflower–SuCMoV compatible interaction, decreases in $CO_2$ fixation rates and increased carbohydrate accumulation were observed (Arias et al., 2003). Infections also generated increases in $H_2O_2$ and oxidative stress (Arias et al., 2005).

Virus infections that produce chlorotic symptoms affect photosynthetic capacity (Lehto et al., 2003; Rahoutei et al., 2000) and carbohydrate metabolism (Técsi et al., 1996). Physiological and biochemical studies indicate that photosynthesis is feed-back-regulated by carbohydrate metabolites (Sheen, 1990; Stitt et al., 1991). Sugars can also regulate respiration rates, storage compound conversion, and source–sink relations (Sheen, 1990) and may act as signal molecules under normal and stressful conditions since plants are able to sense sugar concentration changes (Loreti et al., 2001).

It has not been evaluated whether the increased carbohydrate accumulation observed after SuCMoV infections may also participate in symptom expression. The rationale for this approach is that SuCMoV infection chlorotic mottling symptoms and senescence show common physiological disturbances, such as photosynthesis decrease, chlorophyll degradation and increased sugar accumulation. Sugar accumulation in non-senescent leaves and the supply of external sugars can induce senescence symptoms (Krapp and Stitt, 1994; Wingler et al., 1998), glucose and fructose accumulate markedly during *Arabidopsis thaliana* leaf senescence (Wingler et al., 2006), and glucose and sucrose can repress the transcription of photosynthetic genes (Sheen, 1990).

Alterations in electron transport, resulting from altered photosynthetic activity may generate reactive oxygen species (ROS), such as singlet oxygen, superoxide radical, hydrogen peroxide, and hydroxyl radical (Asada, 1999), a common response of plants to stress conditions. In incompatible plant–pathogen interactions, the key role of ROS/antioxidants relationship has been widely stud-

*Abbreviations:* ΦPSII, quantum efficiency of PS II photochemistry; APX, ascorbate peroxidase; BS, before symptom expression; CAT, catalase; DGC, test of Di Rienzo, Guzmán and Casanoves statistical test in InfoStat; DPI, diphenyl iodonium; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; ES, early symptom expression; EDTA, ethylenediaminotetraacetic acid; EGTA, ethylene glycol-bis-(2-aminoethyl)-N,N,N′, N′-tetraacetic acid; Fv/Fm, maximal photochemical efficiency; G, glucose; GR, glutathione reductase; LS, late symptom expression; PEG, polyethylene glycol; PPV, *Plum pox virus*; PVPP, polyvinylpolypyrrolidone; SuCMoV, Sunflower chlorotic mottle virus; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; S, sucrose; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; Sor, sorbitol; TCA, trichloroacetic acid; XTT, Na,3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium](4-methoxy-6-nitro) benzenesulfonic acid hydrate.

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ied (Lamb and Dixon, 1997), though much less is known about compatible interactions (Riedle-Bauer, 2000). In general terms, apoplastic ROS have been suggested to be generated initially by plasma membrane NADPH oxidases and cell wall peroxidases in response to incompatible pathogen infections (Torres et al., 2006). It has recently been suggested that chloroplasts can be a source of oxidative stress during compatible interactions (Díaz-Vivancos et al., 2008; Song et al., 2009). ROS can induce degradation of key chloroplastic components, e.g. thylakoid proteins (Casano et al., 1994), Calvin cycle enzymes (Asada et al., 1998), pigments, and membrane lipids (Asada and Takahashi, 1987) and thus contribute to virus infection symptom generation. On the other hand, increasing evidence indicates that ROS are signaling molecules, which modulate processes like local and systemic responses to biotic and abiotic stress, hormones signaling pathways, growth, senescence and cell death (Mittler et al., 2004). The dual role of ROS, as toxic or signal molecules, is determined by the rates and subcellular location of ROS generation and degradation (Mittler et al., 2004).

The plant antioxidant system, composed of both enzymatic and non-enzymatic elements, controls ROS level. Superoxide dismutase (SOD) (EC-1.15.1.1), ascorbate peroxidase (APX) (EC-1.11.1.11), catalase (EC-1.11.1.6), and glutathione reductase (GR) (EC-1.6.4.2) are key antioxidant enzymes that modulate the concentration of two of the Haber/Weiss and Fenton reaction substrates, $O_2^{\bullet -}$ and $H_2O_2$, preventing the formation of the highly toxic $OH^{\bullet}$ radical (Asada, 1999). SOD catalyses the disproportionation of $O_2^{\bullet -}$ to $H_2O_2$. Degradation of $H_2O_2$ in the chloroplasts and in the cytosol is carried out by the ascorbate/glutathione cycle, which involves APX and GR activities (Foyer and Halliwell, 1976). APX has chloroplastic and cytosolic isoforms, and catalyses the conversion of $H_2O_2$ to water using ascorbate as electron donor. Approximately 80% of SOD, GR, and APX activities are located in the chloroplast (Asada, 1999). The response of the antioxidant system had been initially evaluated in incompatible plant–pathogen interactions (Lamb and Dixon, 1997), and only recently in systemic infections. A study addressing physiological changes observed at late stages of infection (LS) indicated increases in antioxidant system activity (Riedle-Bauer, 2000). Research works involving earlier infection phases suggest that the observed response of the antioxidant system depends on the pathosystem and also on the infection stage (Clarke et al., 2002; Díaz-Vivancos et al., 2008; Song et al., 2009).

In this work, we examined the potential role of sugars as SuCMoV symptom modulators and their association with photoinhibition and ROS generation/degradation in the compatible sunflower–SuCMoV interaction at various stages of infection: before (BS), at early (ES) and late (LS) symptom expression stages.

## Material and methods

### *Plant material*

Sunflower (*Helianthus annuus* L.) line L2 seeds were provided by Advanta Semillas SAIC, Balcarce, Argentina. Seeds were sown in pots with sterile soil and cultivated in a growth chamber under controlled 16/8 h photoperiod at 250 μmol photon $m^{-2}$ $s^{-1}$, 25 °C, and 65% humidity. The SuCMoV isolate was maintained in *Nicotiana occidentalis* L. and symptomatic leaves were freeze-dried and kept at −20 °C. Sunflower plants at the vegetative stages V1–V2 (Schneiter and Miller, 1981) were rub-inoculated on the upper surfaces of both leaves with an infected leaf homogenate (1:5 w/v in 0.05 M $Na_2HPO_4$, pH 7.5) using carborundum mesh 600 as abrasive. Mock-inoculated plants were used as controls. Samples were always taken from the second leaf pair 4 days post-inoculation (before symptom expression, BS), 7 days post-inoculation (early symptom expression, ES), and 12 days post-inoculation (late symptom expression, LS).

### *Leaf extracts*

Frozen leaf samples (100 mg fresh weight) were ground to a fine powder with liquid nitrogen and homogenized in 50 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1% PVPP (polyvinylpolypyrrolidone). Homogenates were centrifuged at 16,000 × *g* at 4 °C for 25 min and the supernatant was used to determine protein concentration and enzyme activity.

### *Protoplast isolation*

Leaves from inoculated and control plants were surface sterilized with 1% hypochlorite, cut into small segments and incubated with a digestion mixture containing 1% cellulase (Onozuka RS), 0.25% macerozyme (Onozuka R-10), solubilized in 500 mM sorbitol, and 20 mM MES buffer pH 5.8 (Solution A) at 28 °C for 16 h. The protoplast suspension was washed with Solution A followed by centrifugation and re-suspended in Solution A with 10 mM $CaCl_2$. Protoplasts were counted in a Neubauer chamber and diluted to $1 \times 10^5$ per mL.

### *Chlorophyll content*

Chlorophyll content was determined spectrophotometrically at 654 nm in acetonic extracts (Wintermans and De Mots, 1965).

### *Total soluble sugars and starch*

Extracts were obtained following Guan and Janes (1991): 2 g of frozen tissue were ground in 2 mL buffer containing 50 mM HEPES–KOH (pH 8.3), 2 mM EDTA, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM DTT (dithiothreitol). The extract was centrifuged at 15,000 rpm at 4 °C for 15 min and the supernatant was used for soluble sugar determination. Total soluble sugars were measured with anthrone (Fales, 1951) using sucrose as standard. Starch was determined in the pellet, as reducing sugars released after hydrolysis with α-amyloglucosidase (Sumner and Somers, 1944) using glucose as a standard.

### *Sucrose, glucose, fructose and trehalose determination*

Frozen leaf samples (1 g fresh weight) were ground to a fine powder with liquid nitrogen and homogenized, suspended in 2 mL ethanol 80%, and kept at 80 °C for 20 min. The extract was centrifuged at 12,000 × *g* for 10 min. Soluble sugars were extracted three times with hot ethanol. Ethanol was evaporated to 60 °C overnight and re-suspended in 0.5 mL distilled $H_2O$. Sucrose, glucose, fructose and trehalose were determined by HPLC (Shimadzu) using an amine column, isocratic acetonitrile:water (81:19) flow (1 mL/min), at 30 °C. Sugars were identified by their retention times and quantified according to standards.

### *Sugar treatments*

The second pair of leaves from healthy plants was cut and incubated for 24 h in a Petri dish with either water ($H_2O$), 200 mM sugar solutions: glucose (G), sucrose (S) and sorbitol (Sor) under 16/8 h photoperiod at 250 μmol photon $m^{-2}$ $s^{-1}$, 25 °C. Protoplasts were incubated in the same sugar concentration under 80 μmol photon $m^{-2}$ $s^{-1}$ light for 4 h.

### *Chlorophyll fluorescence parameters*

Quantum efficiency of PSII photochemistry under ambient light conditions (250 μmol photon $m^{-2}$ $s^{-1}$, 25 °C) (ΦPSII) and maximum quantum yield (Fv/Fm) in dark-adapted plants (at least