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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 1×10^5 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

30 min) were measured using a pulse amplitude modulated fluorometer (FMS2, Hansatech Instruments, Pentney King's Lynn, UK). Measurements were made on the second pair at BS, ES, and LS stages of infection and in the second pair of leaves from healthy plants incubated for 24 h in a Petri dish with water (H_2O), 200 mM sugar solutions: glucose (G), sucrose (S), or sorbitol (Sor) and solutions of 10% polyethylene glycol (PEG) and maintained at 25 °C under a 16/8 h photoperiod of 250 μmol photon m^{-2} s^{-1}.

Protein

Soluble and membrane proteins were estimated according to Bradford (1976) and Lowry et al. (1951), respectively, in a reaction including 1% sodium dodecyl sulfate (SDS). Bovine serum albumin was used as standard for the calibration curves.

Protein immunoblots

Samples were separated by SDS-PAGE using 12% acrylamide gels. After electrophoresis, the gels were electroblotted to nitrocellulose membranes (Hybond-ECL, Amersham Bioscience) and immunoblot analysis was performed with standard techniques using conjugated alkaline phosphatase and the following antibodies: anti-large Rubisco subunit (RbcL), anti-beta subunit of ATP complex (AtpB), anti-PsbA (D1 protein of photosystem II), anti-PsaC (protein of photosystem I) (AgriSera Co., Sweden) and NDH-F, a subunit of chloroplast Ndh complex (kindly provided by Prof. Sabater, University of Alcalá, Spain).

Apoplastic superoxide radical ($O_2^{\bullet -}$)

Generation of O_2^- was determined histochemically in detached leaves infiltrated with a 0.01% nitro blue tetrazolium (NBT) solution and incubated in the dark for 2 h. Color images were transformed to black and white 8-bit images, and formazan color intensity was determined by an image processing software (Optimas 6.1, Optimas Corporation, Bothell, WA).

O_2^- release from protoplasts to the medium was determined spectrophotometrically, using 1 mM XTT (Na,3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium](4-methoxy-6-nitro) benzenesulfonic acid hydrate) (Frahry and Schopfer, 2001). Samples were read at 470 nm in a spectrophotometer (Beckman DU Series 600, Beckman Instruments, Fullerton, CA) and the activity at 4 h (T1, treatment duration) expressed as ratio T1/T0, were T0 is the activity detected before treatment, at time 0 (Robert et al., 2009).

Antioxidant enzyme activities

Total SOD activity was assayed by measuring the inhibition of the photochemical reduction of NBT at 560 nm (Beauchamp and Fridovich, 1973). Catalase activity was determined by measuring the decrease at 240 nm due to H_2O_2 degradation (Gallego et al., 1996). Ascorbate peroxidase activity was measured according to (Nakano and Asada, 1981) by measuring the H_2O_2-dependent oxidation of ascorbate at 290 nm (the extract medium for this enzyme contained 5 mM ascorbate). Glutathione reductase activity was determined from the rate of NADPH oxidation by the NADPH decrease at 340 nm (Schaedle and Bassham, 1977).

Statistics

Experiments were replicated at least three times. Statistical significances of mean differences were estimated with a DGC test model using InfoStat (InfoStat/Profesional ver. 2007p, Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina).

Results

Chlorophyll, leaf growth, carbohydrate and starch content during SuCMoV infection

SuCMoV infection caused significant decreases in total chlorophyll content in the second leaf pair only at LS stage (Fig. 1A). However, leaves from infected plants were smaller than those controls since early symptom expression (ES) (Fig. 1B), and showed

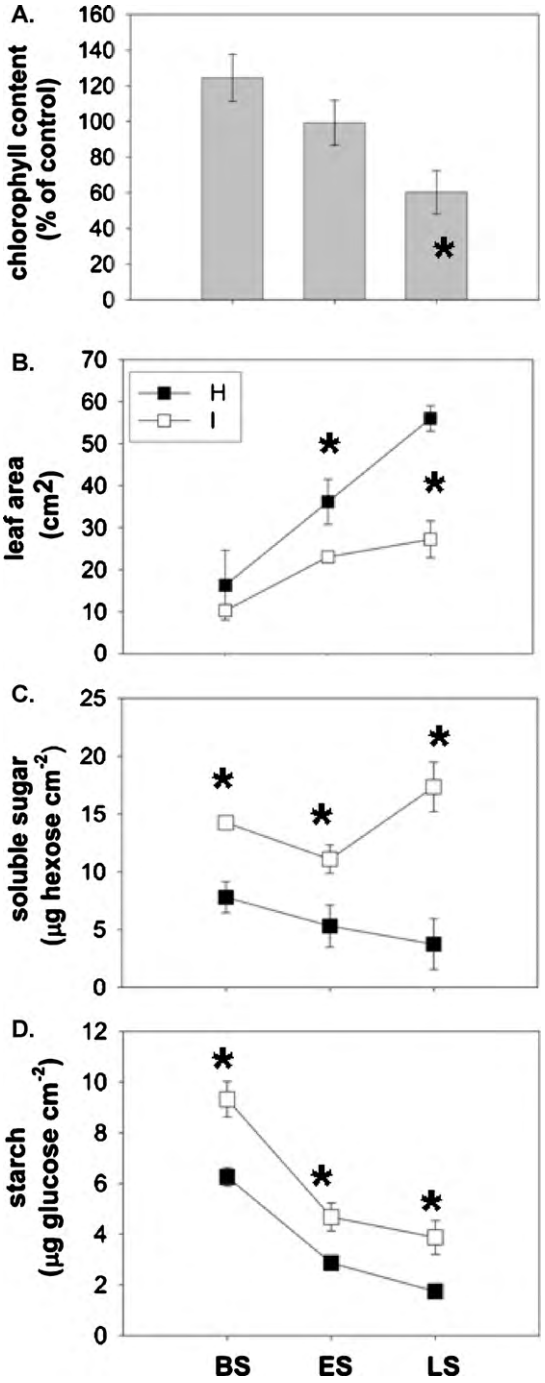

Fig. 1. (A) Chlorophyll content (as % of controls), (B) leaf area, (C) soluble sugar accumulation, and (D) starch accumulation in second pair of leaves from SuCMoV-inoculated (I) and mock-inoculated (H) plants at three stages of SuCMoV infection—BS: before symptom expression, ES: early symptom expression, LS: late symptom expression. Results are means from 12 plants ±SE of four independent experiments. Asterisks indicate significant differences with controls ($p < 0.05$, DGC).

Table 1
Changes in leaf sugar contents (mg g^{-1} FW) during the course of SuCMoV infection, BS: before symptom expression, ES: early symptom expression, LS: late symptom expression.

	BS		ES		LS	
	Healthy	Inoculated	Healthy	Inoculated	Healthy	Inoculated
Sugar content (mg g^{-1} FW)						
Glucose	3.31 ± 1.36	3.01 ± 1.31	8.58 ± 1.25	9.27 ± 1.03 (1.08)	2.64 ± 1.54	9.89 ± 3.93* (3.75)
Fructose	3.6 ± 0.76	7.87 ± 0.28 (2.18)	3.14 ± 0.9	6.38 ± 2.5 (2.03)	4.42 ± 0.90	24.27 ± 7.54* (5.5)
Sucrose	2.85 ± 0.66	7.09 ± 2.16* (2.48)	2.2 ± 0.73	8.42 ± 3.01* (3.83)	2.29 ± 0.04	8.6 ± 3.6* (3.75)
Trehalose	nd	nd	nd	nd	nd	10.62 ± 4.78
Total	9.76 ± 0.82	17.97 ± 4.64* (1.8)	13.92 ± 3.54	24.07 ± 5.9* (1.7)	9.35 ± 2.5	53.38 ± 19.5* (5.7)

nd: not detected. Results are expressed as means ± SE of three independent experiments. Values in parenthesis are fold values as compared to healthy controls.
* Significant differences respect to controls ($p < 0.05$, DGC).

increased soluble sugar and starch content, even before symptoms became evident (BS, Fig. 1C and D, respectively) these changes were more marked as infection progressed (Fig. 1C). HPLC analyses indicated that increases in sucrose and fructose were mainly responsible for the soluble sugar increase observed from BS, while glucose and trehalose also increased, but later, at LS (Table 1). The early increase in sugar concentration at BS led us to study the role of sugars as modulators of chlorotic mottling induced by SuCMoV.

Chlorophyll fluorescence and photosynthetic proteins

A significant decrease in ΦPSII, the quantum efficiency of photosystem II photochemistry from light-adapted plants and in Fv/Fm, the maximum efficiency of photosystem II from dark-adapted plants, was detected from the initial stages of virus infection (BS, Table 2), indicating that photoinhibitory processes are early symptoms of SuCMoV infection and continue throughout it. Incubation of healthy leaves in glucose or sucrose lead to similar changes in ΦPSII and Fv/Fm (Table 2). Sorbitol and PEG, used as osmotic controls, also induced ΦPSII decreases, however, did not affect Fv/Fm (Table 2).

To further describe the effects of SuCMoV infection and sugars on photosynthesis, the level of some key photosynthetic proteins was analyzed by protein immunoblot: PsaC, a component of PSI; D1, an essential part of PSII core whose degradation indicates photoinhibition; AtpB, a subunit of ATPase complex; the NDH-F subunit of chloroplastic Ndh complex and the Rubisco large subunit. The level of D1 protein was clearly reduced in infected leaves at BS (Fig. 2A) whereas NDH-F and AtpB increased. On the other hand, PsaC and Rubisco were not affected at this stage. At LS, the effect of SuCMoV infection on D1 presence was even greater, Rubisco also showed a marked decrease, and NDH-F, AtpB, and PsaC remained unchanged (Fig. 2A).

The effect of sugars on these photosynthetic proteins was studied in healthy leaves incubated with sucrose, glucose or sorbitol for 24 h. RuBisCo, D1 and PsaC protein levels decreased with the sucrose treatment, whereas the other proteins did not show significant changes (Fig. 2B).

Table 2
Effects of SuCMoV infection, sucrose, glucose, sorbitol and PEG treatment on Fv/Fm and ΦPSII.

Treatment	Fv/Fm	ΦPSII
BS		
H	0.880 ± 0.001	0.757 ± 0.002
I	0.874 ± 0.002*	0.748 ± 0.002*
ES		
H	0.877 ± 0.001	0.760 ± 0.002
I	0.868 ± 0.002*	0.754 ± 0.002*
LS		
H	0.882 ± 0.002	0.764 ± 0.002
I	0.866 ± 0.005*	0.753 ± 0.004*
H_2O	0.860 ± 0.0044	0.753 ± 0.005
Glu	0.826 ± 0.008*	0.715 ± 0.008*
Suc	0.832 ± 0.007*	0.717 ± 0.012*
Sor	0.851 ± 0.003	0.710 ± 0.008*
PEG	0.861 ± 0.006	0.690 ± 0.01*

Mock inoculated (H) and SuCMoV inoculated (I). Plants before symptom expression (BS), early symptom expression (ES), late symptom expression (LS). Healthy leaves incubated for 24 h with either water, 200 mM sugar solutions: glucose (G), sucrose (S) and sorbitol (Sor), 10% PEG under a 16/8 h photoperiod of 250 μmol photon m^{-2} s^{-1}, at 25 °C. Results are expressed as means ± SE of four independent experiments.
* Significant differences respect to controls ($p < 0.05$, DGC).

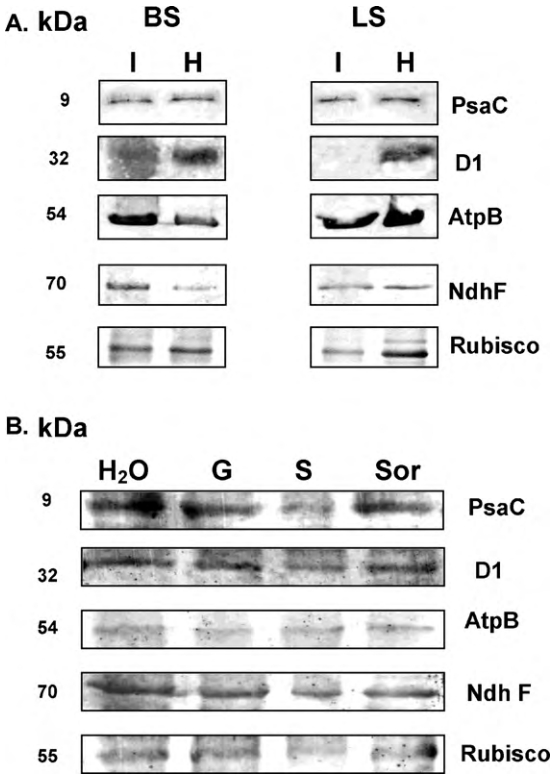

Fig. 2. Immunoblots of chloroplast proteins: D1 (PsbA of PSII), PsaC (PSI), AtpB (subunit B of ATPase), NdhF (Ndh complex) and large subunit of Rubisco. (A) Chloroplastic proteins during infection, before symptom expression (BS) and late symptom expression (LS), second pair of leaves from SuCMoV-inoculated (I) and mock-inoculated (H) plants. (B) Chloroplastic proteins from healthy leaves incubated for 24 h with either water or 200 mM sugar solutions: glucose (G), sucrose (S) and sorbitol (Sor) under a 16/8 h photoperiod of 250 μmol photon m^{-2} s^{-1}, at 25 °C.

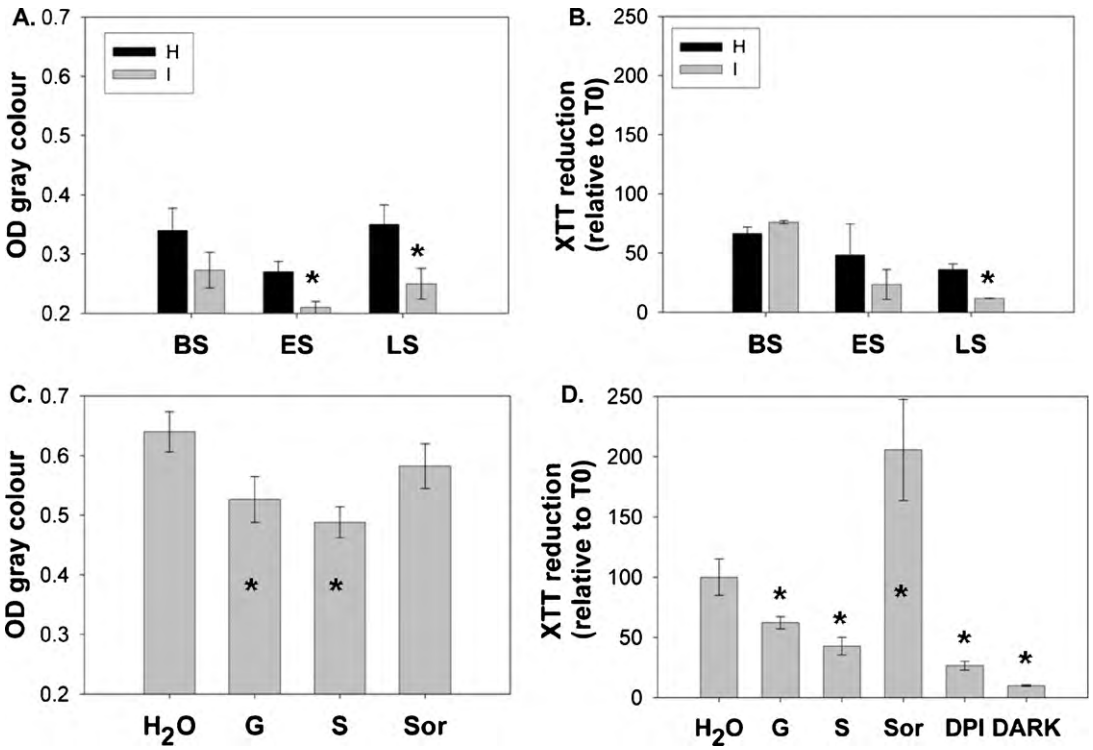

Fig. 3. Apoplastic superoxide radical accumulation. (A) Second pair of leaves from SuCMoV-inoculated (I) and mock-inoculated (H) plants at three stages of SuCMoV infection—BS: before symptom expression, ES: early symptom expression, LS: late symptom expression. (B) Protoplasts from healthy (H) and inoculated (I) leaves, incubated for 4 h under 80 μmol photon m^{-2} s^{-1}, at 25 °C. (C) Healthy leaves incubated for 24 h with either water or 200 mM sugar solutions: glucose (G), sucrose (S) and sorbitol (Sor) under a 16/8 h photoperiod of 250 μmol photon m^{-2} s^{-1}, at 25 °C. (D) Protoplasts of healthy leaves incubated for 4 h in the dark at 25 °C, or in the light (80 μmol photon m^{-2} s^{-1}) with H_2O, 200 mM G, S, Sor or 50 μM DPI. Results are expressed as means of ±SE of four independent experiments. Asterisks indicate significant differences with controls ($p < 0.05$, DGC).

ROS generation/degradation during infection

Apoplastic superoxide generation

Apoplastic superoxide radical generation, determined by histochemical assays, significantly decreased in SuCMoV-infected leaves at ES and LS (Fig. 3A). Protoplasts isolated from infected leaves showed the same effect, as determined by following the reduction of XTT in the incubation medium (Fig. 3B). Incubation with sucrose and glucose mimicked this effect, both in healthy leaves (Fig. 3C) and in protoplasts (Fig. 3D). The inhibitory effect of these sugars on apoplastic superoxide radical generation was not due to their osmotic pressure, because apoplastic superoxide radical generation markedly increased in both leaves and protoplasts incubated with a sorbitol solution of equal osmolarity. The presence of DPI, a suicide inhibitor of the NADPH oxidase complex in the protoplast incubation medium inhibited XTT reduction to 60% (Fig. 3D).

Antioxidant enzyme activities

SOD, APX and GR activities were higher in infected leaves than in controls at all post-inoculation times (Fig. 4A.1, C.1 and D.1, respectively). The activities of GR and APX, which are key enzymes of the Asada–Halliwell cycle, the main mechanism to remove hydrogen peroxide from the chloroplasts, significantly increased in infected leaves (Fig. 4C.1 and D.1). CAT activity only increased at ES stage, decreasing at LS stage (Fig. 4B.1). Sugar treatments induced very similar changes on antioxidant enzyme activities in healthy leaves. Both sucrose and glucose induced significant increases in SOD, APX and GR activities (Fig. 4A.2, C.2 and D.2, respectively), whereas CAT activity was markedly and significantly reduced by both sugars (Fig. 4B.2).

Discussion

Chlorotic mottling symptoms caused by SuCMoV become evident several days after initial infections, at ES, and are initially expressed as chlorotic yellow bright pin points that later coalesce (Lenardon et al., 2001) and at LS lead to significant losses in chlorophyll (Fig. 1A). Physiological changes that lead to symptom expression happen since the early phases of infection. Early increases in soluble sugars and starch (Fig. 1C and D), photoinhibition (Table 2) and increases in some antioxidant enzymes (Fig. 4) are some of the changes observed before symptom expression (BS). Some of these changes had been reported before for SuCMoV infections in sunflower (Arias et al., 2003, 2005), and since increases in carbohydrates were accompanied with a reduction in carbon fixation, it was suggested that carbohydrate increase was originated from sources not directly related to current carbon fixation, such as gluconeogenesis, increased carbon import or decreased export, as more recently reviewed by Berger et al. (2007). It had not been investigated before, specifically, whether increased sugar accumulation can affect other physiological changes observed during symptom development.

In this work we detected decreases in ΦPSII and Fv/Fm, which occur at the early stages of infection (BS, Table 2), as other authors have also reported (Berger et al., 2007). ΦPSII measures the proportion of the light absorbed by chlorophylls associated with PSII that is used in photochemistry. This parameter gives a measure of the rate of linear electron transport and it is an indication of overall photosynthesis. Fv/Fm is a measure of the maximum or potential efficiency of PSII, and it is a sensitive indicator of photoinhibition induced by PSII damage (Maxwell and Johnson, 2000). The term photoinhibition applied to fluorescence analysis, refers to

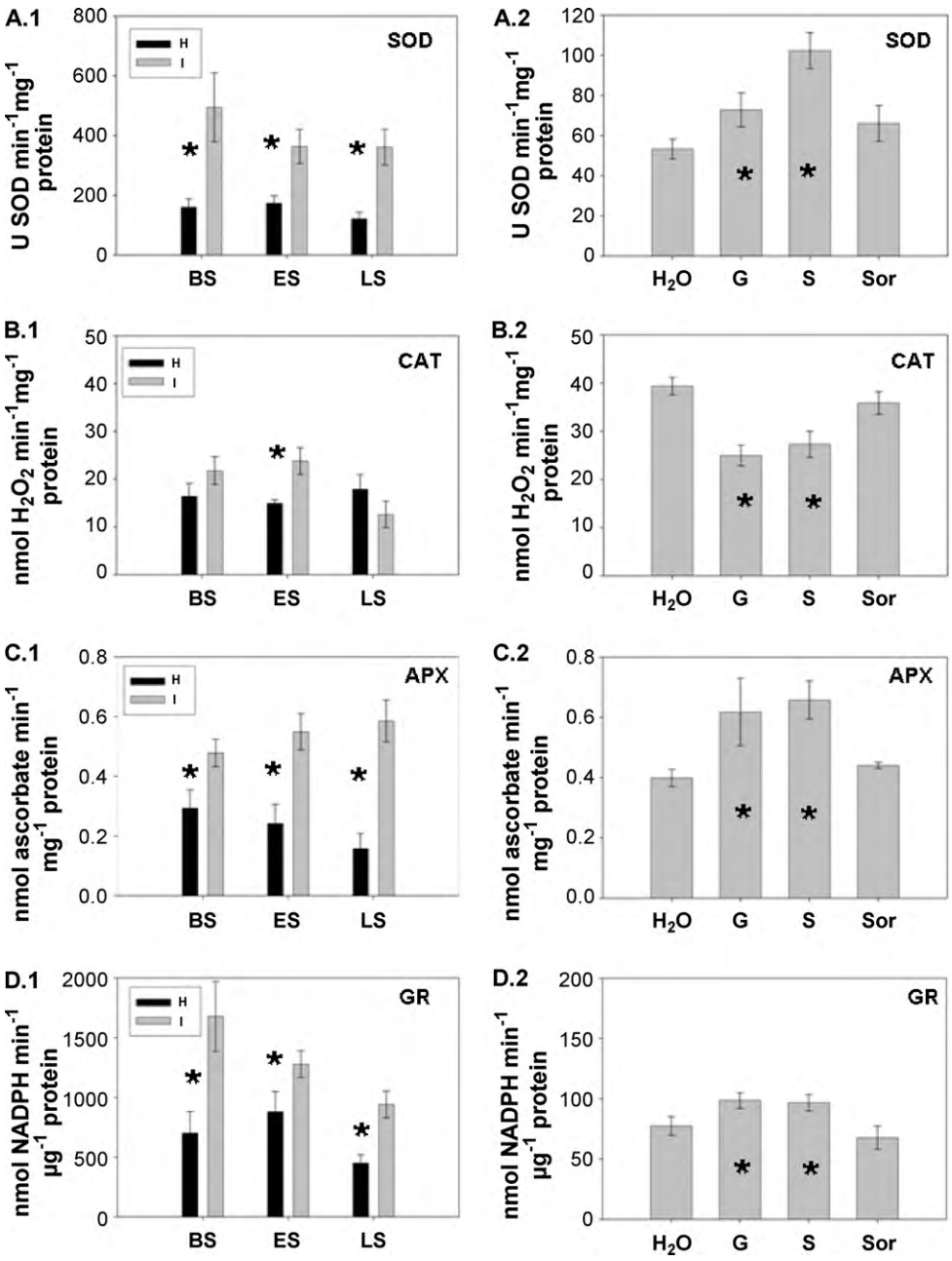

Fig. 4. Antioxidant enzyme activities in sunflower leaves. (A) Superoxide dismutase (SOD), (B) catalase (CAT), (C) ascorbate peroxidase (APX), (D) glutathione reductase (GR). (1) Second pair of leaves from SuCMoV-inoculated (I) and mock-inoculated (H) plants at three stages of SuCMoV infection—BS: before symptom expression, ES: early symptom expression, LS: late symptom expression and (2) healthy leaves incubated for 24 h with either water or 200 mM sugar solutions: glucose (G), sucrose (S) and sorbitol (Sor) under a 16/8 h photoperiod of 250 μmol photon m^{-2} s^{-1}, at 25 °C. Results are expressed as means of ±SE of four independent experiments. Asterisks indicate significant differences with controls ($p < 0.05$, DGC).

both reversible inhibition protective mechanisms and to photooxidative damage to the reaction center of PSII (Osmond, 1994). We observed that the early decreases in ΦPSII and Fv/Fm induced by SuCMoV correlated with a decrease in the level of D1 protein, which is an essential protein of PSII core and whose degradation is an indicator of photoinhibition with photodamage. Virus infection effects on D1 had been reported in tobacco plants 20 days after infection with flavum strain of *Tobacco mosaic virus* (Lehto et al., 2003). Photoinhibition, D1 and Rubisco decrease and sugar level increase in infected leaves continued throughout the infection process. Sugar treatments induced similar effects to those of SuCMoV infection on ΦPSII, Fv/Fm and D1 and Rubisco protein levels. Even though, similar effects on ΦPSII were induced by sugars and their osmotic controls (sorbitol and PEG), the latter did not affect either Fv/Fm or D1 protein levels, suggesting that sugars may specifically induce a photoinhibitory process related with the integrity of PSII. These results also support a senescence-promoting role for sugars in sunflower leaves. Evidence supporting the notion that sugar signaling plays a role in senescence regulation in combination with other signals resulting from biotic or abiotic stress has been discussed by Wingler and Roitsch (2008).

While D1 protein levels decreased in infected leaves, other proteins involved in photosynthesis, such as PsaC, a component of PSI, showed no changes, whereas AtpB, a subunit of ATPase complex, and NDH-F, a subunit of chloroplastic Ndh complex, showed increased levels at BS stage. It is apparent that photoinhibited

chloroplasts from infected leaves preserved their machinery to transfer electrons and generate ATP through a cyclic or pseudocyclic flux.

Apoplastic superoxide level was markedly decreased by SuCMoV infection since ES stage, 7 days after inoculation. Apoplastic ROS production was partially inhibited by DPI suggesting the NADPH oxidase complex participation in this system (Bolwell et al., 2002). Nevertheless, it has been proposed that in addition to NADPH oxidases, pH dependent cell wall peroxidases, germin-like oxalate oxidases and amine oxidases can generate ROS at the apoplast (Mittler et al., 2004) and should also be considered in this context.

The ROS–antioxidant interaction determines, to a large extent, the cellular redox state, which plays a central role in environmental stress perception and defence response modulation (Foyer and Noctor, 2005; Mittler et al., 2004). Higher SOD, GR, and APX total activities were found in infected leaves along the infection process, while CAT increased at ES. Díaz-Vivancos et al. (2008) described changes in antioxidant enzyme activities in pea plants (cv. Alaska) at two stages of infection by *Plum pox virus* (PPV) and found similar responses. They proposed a possible role for peroxisomes as a putative source of ROS generation during the phase of disease development in PPV-infected pea plants and their results suggest that responses seem to be pathosystem and stage-specific.

Very similar responses of apoplastic ROS and the antioxidant system were found when healthy leaves were treated with sugars (Figs. 3 and 4), decreases in apoplastic superoxide accumulation, increases in SOD, GR and APX activities were observed. This is in agreement with the proposed dual role sugars seem to have in relation to ROS: they can be involved in ROS-producing metabolic pathways but can also contribute to ROS scavenging mechanisms (Couée et al., 2006).

In SuCMoV infections, increased superoxide presence was transiently observed 6 h after inoculation (Arias et al., 2005), but its source was not investigated. It is proposed that high sugar concentration resulting from SuCMoV infection may have negatively affected photosynthetic carbon fixation through a feed-back mechanism (Sheen, 1990; Stitt et al., 1991) leading to photoinhibition (Jang and Sheen, 1994) and increased ROS production in chloroplasts (Díaz-Vivancos et al., 2008; Song et al., 2009). It is this initial increase in chloroplastic ROS which may subsequently lead to a stimulation of antioxidant activity (Mittler, 2002) which ultimately results in reduced ROS not just in chloroplasts but also in other compartments, such as the apoplast. It has been shown that apoplastic ROS generation under photooxidative stress conditions may be dependent on chloroplastic ROS production (Robert et al., 2009) or ROS-related signals from chloroplast (Yaeno et al., 2004). Alternatively, the transient increase in ROS observed in leaf 2, a non-inoculated leaf, 6 h after SuCMoV inoculation to leaf 1, may be an early consequence of infection (Love et al., 2005; Torres et al., 2006), and ROS, acting as signals in this case, may induce increased antioxidant activity (Mittler, 2002), which subsequently mitigates ROS. At that stage, the virus has not yet arrived to leaf 2, as demonstrated by leaf-excision experiments (Rodriguez et al., unpublished). Virus arriving at leaf 2 turn it into a strong sink for carbohydrate import (Berger et al., 2007) and may also stimulate gluconeogenesis, increasing soluble sugars which, in turn, may inhibit photosynthesis, thus contributing in a synergistic way to symptom development through the process described earlier in this paragraph. A detailed kinetic analysis of metabolic changes evoked at the initial stages of virus infection will provide information on these alternatives.

In short, our results suggest that sugar increases during the course of SuCMoV infection may modulate photoinhibitory processes and participate in symptom expression. On the other hand, increased singlet oxygen generation resulting from the photoinhibitory process induced by SuCMoV is to be expected. Singlet oxygen ($O_2{}^{1\bullet}$) is a potent agent of oxidative damage that could also contribute to chlorotic symptom development (Fryer et al., 2002). SuCMoV infection could in turn increase ROS generation in other cellular compartments (Díaz-Vivancos et al., 2008; Song et al., 2009) in association with respiratory and photorespiratory processes. These alternative ROS sources should also contribute to the development of chlorotic symptoms.

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