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Sunflower chlorotic mottle virus in compatible interactions with sunflower: ROS generation and antioxidant response

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Abstract

Sunflower chlorotic mottle virus (SuCMoV) is a recently described potyvirus that causes systemic infections in sunflower plants leading to chlorotic mottling and important growth reductions and yield losses. Oxidative damage is expressed after symptom development in this host-pathogen combination. The involvement of antioxidant enzyme activities in disease susceptibility was studied in two sunflower lines differing in the intensity and rate of development of SuCMoV infections: L2 is more susceptible than L33. A transient superoxide production peak was detected in leaves of both lines before symptom development. H_2O_2 accumulation increased before symptom expression in infected plants of L33 but in L2 such increase was registered only after symptoms became evident. In healthy plants of both lines, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) showed similar activity levels. In inoculated plants of line L2, but not in L33, SOD and CAT activities increased significantly before the appearance of symptoms, and APX increases were detected later. A 1 mM SA treatment effectively decreased SuCMoV accumulation in plants of L2 but it did not affect it in L33. This treatment increased H_2O_2 accumulation and prevented the increase in antioxidant enzyme activities in infected plants of L2. It is suggested that increases in antioxidant enzyme activities interrupted the signals generated by the increase in ROS, which may have otherwise triggered defence reactions in the host and thus, resulted in a compatible interaction.

Abbreviations: APX – ascorbate peroxidase; CAT – catalase; MDA – malondialdehyde; ROS – reactive oxygen species; SOD – superoxide dismutase; SuCMoV – *Sunflower chlorotic mottle virus*

Introduction

Sunflower chlorotic mottle virus (SuCMoV) is a recently described potyvirus that causes systemic infections in sunflower plants leading to chlorotic mottling, plant stunting and important yield losses (Dujovny et al., 1998; Dujovny et al., 2000; Lenardon et al., 2001). Virus infections causing mosaic or mottling symptoms in leaves are often accompanied by physiological disturbances such as increased carbohydrate accumulation (Técsi et al., 1994; Sindelárová et al., 1999) altered carbon fixation and carbohydrate partitioning (Goodman et al., 1986; Balachandran et al., 1997; Clover et al., 1999). In the SuCMoV compatible plant-virus interaction, decreased CO_2 fixation rates and increased carbohydrate accumulation were observed after symptom development (Arias et al., 2003). These changes in carbon metabolism can result in altered electron transport (Balachandran et al., 1997), leading to reactive oxygen species (ROS) generation.

ROS are partially reduced O_2 types that participate in development, hormone action, and in responses to biotic and abiotic stresses (Mittler, 2002). In plant cells, ROS, mainly H_2O_2, superoxide

anion ($O^{\cdot}_2{}^{-}$) and hydroxyl radical ($OH^{\cdot}$) are generated in the cytosol, chloroplasts, mitochondria and the apoplastic space (Bowler and Fluhr, 2000; Mittler, 2002). ROS participate in signaling events that regulate ion channel activity (Foreman et al., 2003) and gene expression (Neill et al., 2002), affect the rheological properties of cell walls (Cosgrove, 1999), and are also responsible for oxidative damage. The latter role, a negative consequence of ROS presence, has led to extensive studies of the plant antioxidant system (Mittler, 2002), which includes molecules such as ascorbate and glutathione and the activity of enzymes such as superoxide dismutase, catalase and ascorbate peroxidases (Asada, 1994; Inzé and Van Montagu, 1995).

ROS generation is a common feature in both incompatible and compatible plant-pathogen interactions (Bolwell et al., 1998; Bolwell et al., 2002). The oxidative burst observed in the initial stages of incompatible interactions (Dangl et al., 1996; Hammond- Kosack and Jones, 1996) is responsible for the induction of defence reactions leading to hypersensitive responses (Low and Merida, 1996) and the development of systemic acquired resistance (SAR) (Sandermann, 2000). Less attention has been devoted to the role of ROS in compatible plant-pathogen interactions (Riedle-Bauer, 2000; Stone et al., 2000; Venisse et al., 2001). The activities of enzymes involved in the detoxification of ROS in the *Phaseolus vulgaris*-white clover mosaic potexvirus compatible interaction were studied by Clarke et al. (2002). These authors suggested virus replication and disease development were favoured when those antioxidant enzymatic activities decreased. On the other hand, an induction of superoxide dismutase, catalase, total peroxidase and ascorbate peroxidase activities was observed in the compatible interaction between *Cucumber mosaic virus* and *Zucchini yellow mosaic virus* with *Cucumis sativus* and *Cucurbita pepo* plants, respectively (Riedle-Bauer, 2000).

The availability of sunflower lines differing in the intensity and rate of SuCMoV symptom development (Lenardon et al., 2005) renders the possibility of studying the involvement of antioxidant enzyme activities in disease susceptibility in this pathosystem. Since oxidative damage is expressed after symptom development in this host-pathogen combination (Arias et al., 2003) it was hypothesed that more oxidative damage and lower antioxidant enzyme activities would be found in the susceptible line. The effect of a salicylic acid treatment, which is known to reduce virus replication and movement (Murphy and Carr, 2002), was studied to determine whether alterations in virus accumulation were reflected on antioxidant enzyme activity.

Materials and methods

Two sunflower (*Helianthus annuus*) lines differing in SuCMoV susceptibility were used: line L2 and L33. Line L33 has a resistance gene (Rcmo-1, Lenardon et al., 2005). Seeds were provided by Advanta Semillas SAIC, Balcarce, Argentina. Seeds were sown in pots with sterile soil and grown in a naturally illuminated greenhouse. Supplemental illumination was provided by incandescent lamps. The SuCMoV isolate was maintained in *Nicotiana occidentalis* and symptomatic leaves were freeze-dried and kept at –20 °C. For virus inoculation, those leaves were homogenized (1/5 w/v) in 0.05 M $Na_2H\ PO_4$, pH 7.5 and sunflower plants at the vegetative stage V1 (Schneiter and Miller, 1981) were rub-inoculated on the upper surface of both blades of the first leaf pair with the slurry, using carborundum mesh 600 as abrasive. Control plants were mock-inoculated with buffer and abrasive. Samples were taken three days after inoculation (BS), on the day of symptom appearance (ES), and every three days thereafter, as successive newly developed leaves reached approximately 50% of their final size. ES was day 9 for leaf pair 2 of line L2, and symptoms could then be observed in new leaves as they began to expand. Late samples (LS) were taken from fully expanded leaves 1 month after inoculation.

Serological virus detection

SuCMoV infections were detected by DAS-ELISA (Clark and Adams, 1977) using an antiserum obtained at our Institute (Dujovny et al., 1998). Each plate contained six healthy and two infected control samples. Plants were considered infected when A_{405} readings were higher than the sum of the healthy controls average plus three standard deviations.

Oxidative damage and reactive oxygen species concentration

Oxidative damage in leaves was evaluated by chlorophyll retention and malondiadehyde (MDA) concentration. Chlorophyll and MDA were measured in alcohol extracts according to Tetley and Thimann (1974), and Heath and Packer (1968), respectively. H_2O_2 was estimated in leaf extracts using 3–5 dinitrosalicylic acid, according to Guilbault et al. (1968), including a blank with catalase (EC 1.11.1.6) for each sample. To determine $O^{\cdot}{}_2^-$ accumulation, detached leaves were gently infiltrated (4 min, gentle vacuum was provided by exerting and releasing vacuum every 15 s) with a 0.01% NBT solution in water and incubated for 2 h at 30 °C in the dark, with very slow shaking. Leaves were then mounted on a glass slide, and scanned. Colour images were first inverted to obtain a negative, transformed to black and white 8−bit images, and formazan colour intensity (in the negative corresponding to the lighter tones of grey), determined by image processing software (Optimas 6.1, Optimas Corporation, Bothell, WA). Blue colour intensity was also calculated with this software.

Antioxidant enzyme activities

Frozen leaf samples (100 mg fresh weight) were ground to a fine powder in liquid nitrogen, and homogenized in 50 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1% PVPP (polyvinylpolypyrrolidone), and 5 mM ascorbate in samples for ascorbate peroxidase activity. Homogenates were centrifuged at 16000*g* for 25 min at 4 °C and the supernatant was used to determine protein concentration (Bradford, 1976) and enzyme activity. Total superoxide dismutase (SOD) activity was assayed at 560 nm by measuring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 1973). One unit of SOD activity was defined as the amount of enzyme which causes a 50% inhibition of the photochemical reduction of NBT, and SOD specific activity was expressed as units per mg protein. Catalase (CAT) activity was determined at room temperature by measuring the decrease in A_{240} after adding 5 mM H_2O_2 to samples (Gallego et al., 1996). Total ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981), by measuring the H_2O_2-dependent oxidation of ascorbate at 290 nm. The reaction mixture contained appropriate dilutions of the samples in 50 mM phosphate buffer pH 7.4, 0.5 mM ascorbic acid and 0.1 mM H_2O_2 . The reaction mixture did not contain EDTA, which was observed to inhibit the reaction in sunflower.

Salicylic acid (SA) treatment

A 1 mM aqueous SA acid solution containing 0.05% Tween 20 was applied three to four times per week with a cotton swab on the upper surface of all expanded leaves, during the length of this experiment. This SA concentration was chosen after preliminary experiments showed it could reduce virus concentration and symptom expression without damaging the plants. Control plants were mock-inoculated with a 0.05% Tween 20 solution.

Statistics

Trials were replicated twice and results were subject to analysis of variance using the software Statistica (Complete Statistical Systems, 1992).

Results

SuCMoV accumulation and oxidative damage

Line L2 developed severe chlorotic mottling symptoms while L33 showed a scarce and isolated chlorotic pinpoint (Figure 1). In L2 symptoms appeared in the inoculated leaf after 7–9 days and later, in successively expanded leaves (Figure 2). Symptom appearance in L33 was delayed by at least six days. The virus could not be detected before symptom appearance (Arias et al., 2003). In L2, virus concentration increased rapidly and could be detected in leaf 2, nine days after inoculation, while in line L33 virus could first be detected 15 days after inoculation (Figure 3). The differences in virus accumulation kinetics between the two lines were reflected in symptom development; in L2 chlorophyll retention was already low in leaf 2, the first that expressed symptoms (Figure 4a), whereas in L33 chlorophyll content declined as symptoms developed. Oxidative damage, expressed as increase in MDA concentration,

Figure 1. SuCMoV infection symptoms in leaf 5 from plants of lines L33 (a) and L2 (b). The image was taken 21 days after inoculation.

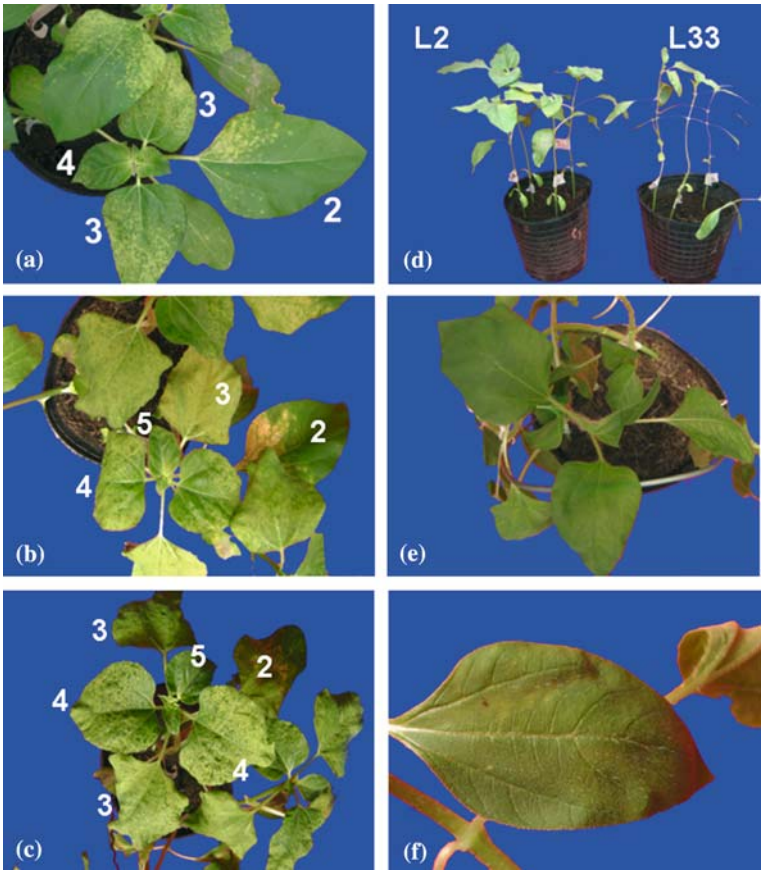

Figure 2. Symptoms in sunflower lines L2 and L33, at various times after inoculation with SuCMoV. (a), (b) and (c): L2, 8, 12 and 14 days after inoculation; numbers indicate leaf pairs. Notice symptom spread as leaves expand. (d): plants of L2 and L33 8 days after inoculation. (e): Plants of L33, 12 days after inoculation, notice the absence of symptoms. (f): faint early symptoms in leaf 2 of L33, 14 days after inoculation.

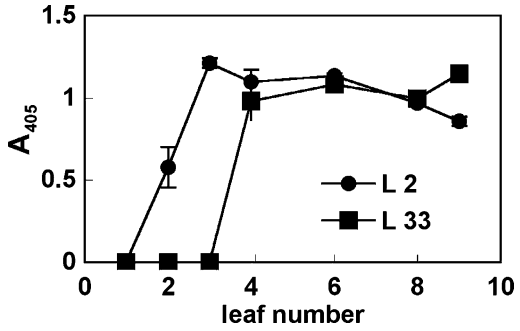

Figure 3. SuCMoV accumulation (expressed as A_{405} values of ELISA reactions) measured in the most recently expanded leaf of two sunflower genotypes differing in disease susceptibility. Measurements were performed in successive leaves. Results are means ± SE of five plants.

was very high when symptoms first became evident in L2 (Figure 4b), whereas in L33 it was initially lower but increased as symptoms developed.

ROS generation and antioxidant enzyme activity in SuCMoV-infected plants

H_2O_2 accumulation increased early in infected plants of L33 (Figure 5) before symptom expression (BS) but in L2 the increase in H_2O_2 accumulation was registered only after symptoms became evident (SE). Though levels were lower in this line than in L33, the effect persisted even at the later stages of infection (LS). In healthy plants of both lines, the three antioxidant enzymes measured in this work showed similar activity levels (Figure 6). In inoculated plants of line L2, SOD and CAT activities increased significantly before symptom expression (BS, Figure 6, a.1 and b.1), and APX increases were detected later (LS, Figure 6 c.1). SOD in L2 peaked at 48 h (Figure 7a). A superoxide production peak was detected 4 h after infection, before symptom development, in leaves from infected plants of both lines. In L2 (Figure 7b), as expected, the increase in SOD activity was displaced in time. The increase in CAT activity could be detected 24 h after inoculation in this line (Figure 7c). Both SOD and CAT activity increases were transient. These changes were not observed in L33 (Figure 6, a.2, b.2 and c.2).

Virus accumulation and antioxidant enzyme activity in responses to SA treatment

The SA treatment was aimed at decreasing virus concentration (Murphy and Carr, 2002). It effectively decreased SuCMoV accumulation in plants of the susceptible line but it did not affect it in the tolerant line (Figure 8). When plants were sampled before symptom expression (BS), SA treatments resulted in decreased SOD activity in healthy and

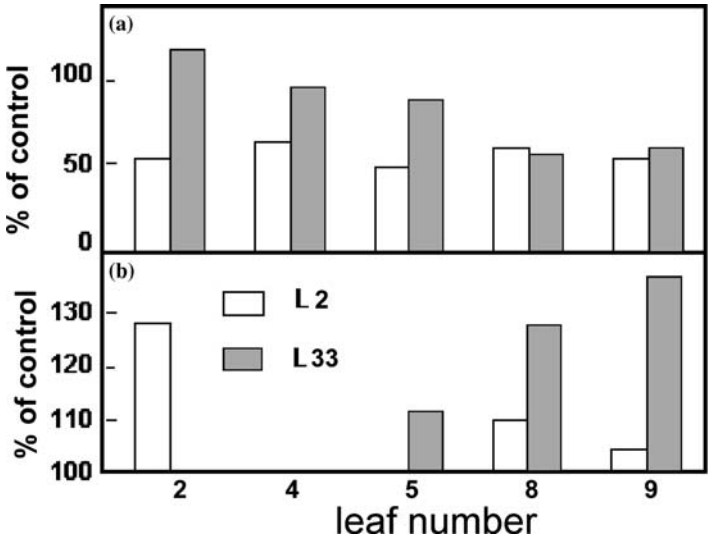

Figure 4. Chlorophyll (a) and MDA (b) concentration in SuCMoV-inoculated sunflower plants, measured in the most recently expanded leaf, and expressed as percentage of healthy control values. Average control values were 0.039 ± 0.002 and 0.038 ± 0.001 mmolesMDA/g FW, for lines L33 and L2, respectively. Symptoms in L2 appeared in leaf 2 and in L33, in leaf 4.

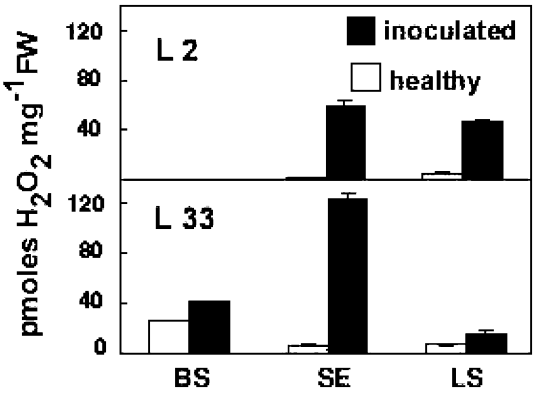

Figure 5. H_2O_2 accumulation in leaves of sunflower lines L2 and L33, at various stages of SuCMoV infection: BS: before symptom expression, SE: initial symptom expression, LS: 21 days after initial symptom expression. Results are means ± SE of four plants.

inoculated plants of both lines (Table 1). However, the effect on inoculated plants of L2 was more evident, as SA-treated plants of this line did not exhibit the sharp increase in SOD or CAT activity (Figure 5), and consequently, an increase in H_2O_2 concentration was detected in salicylic-acid treated plants of L2 (Figure 9). APX activity increased in SA-treated plants, but there was no differential effect on inoculated plants of either line (Table 1). Superoxide production was similar in treated and non-treated plants.

Discussion

Oxidative stress has been observed in the compatible interaction between SuCMoV and sunflower plants (Arias et al., 2003) and results from the present work show that in sunflower lines with different responses to SuCMoV infection, the changes in oxidative stress indicators, such as chlorophyll loss and MDA increase, appear to be associated with virus concentration increase in the tissues (Figures 3 and 4). ROS are products of normal cell metabolism, and increase in response to

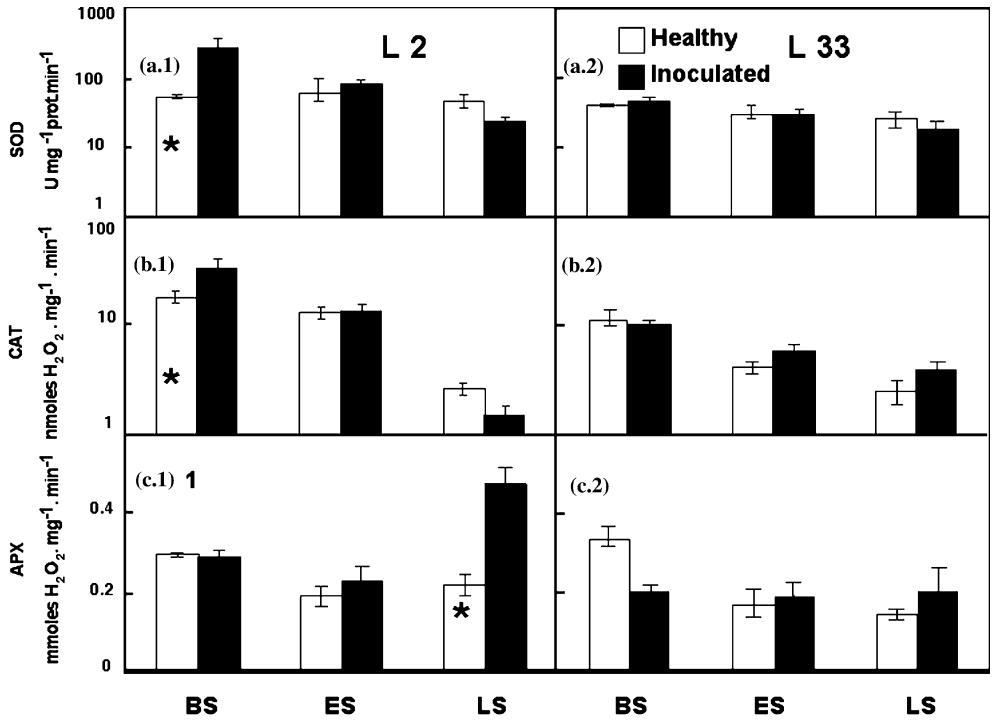

Figure 6. Superoxide dismutase (a.1 and 2: SOD), catalase (b.1 and 2: CAT) and ascorbate peroxidase specific activity (c. 1 and 2: APX) in healthy and SuCMoV-infected plants of two sunflower lines (L2: a.1, b.1 and c.1; L33: a.2, b.2 and c.2), at various stages of virus infection: BS: before symptom expression, SE: initial symptom expression, LS: 21 days after initial symptom expression. Results are means ± SE of four plants. Asterisks denote significant ($P < 0.05$) differences between inoculated and non-inoculated plants.

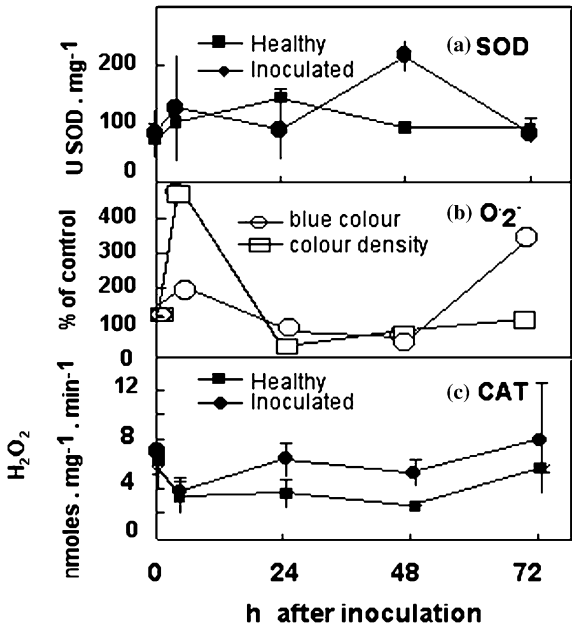

Figure 7. SOD activity (a), superoxide accumulation (b) and catalase activity (c) in leaves of sunflower line L2 at various times after inoculation with SuCMoV (all before symptom expression, BS). In a and c, results for healthy and inoculated plants are shown, in b results are expressed as % of healthy plants. Results are means ± SE of four plants.

environmental stress. Pathogen infections in plants result in increased ROS presence, both in incompatible systems producing hypersensitive responses (Hammond- Kosack and Jones, 1996; Dangl et al., 2000), as well as in compatible reactions associated with systemic disease symptoms produced by viruses, fungi and bacteria (Chai and Doke, 1987; Baker et al., 1993; Kiba et al., 1997; Deighton et al., 1999; Riedle- Bauer, 2000; Venisse et al., 2001).

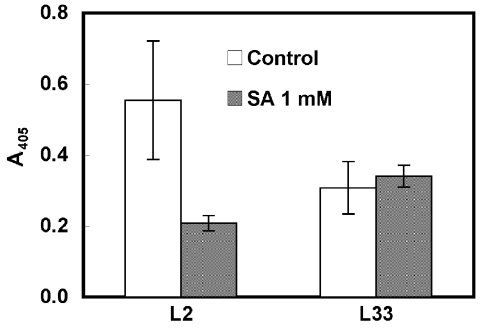

Figure 8. Effect of 1mM SA on SuCMoV accumulation (measured as A 405) in plants of two sunflower lines: L2 and L33. Measurements are means ± SE of five leaves.

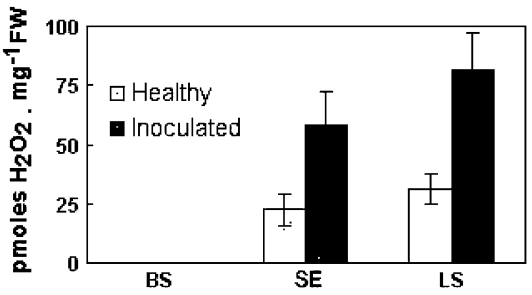

Figure 9. H_2O_2 accumulation in sunflower L2, treated with 1 mM SA, at various stages of SuCMoV infection: BS: before symptom expression, SE: initial symptom expression, LS: 21 days after initial symptom expression. Results are means ± SE of four plants.

It is generally accepted that ROS generation plays an important role in virus resistance in incompatible reactions, participating in the development of the hypersensitive response (HR) (Levine et al., 1994; Mittler, 2002) which results in preventing virus systemic spread. $O^{\cdot}{}_2^-$ participates in the induction of the defense response (Jabs et al., 1996; Grant and Loake, 2000; Sagi and Fluhr, 2001). $O^{\cdot}{}_2^-$ release has also been shown to be necessary for phytoalexin accumulation in *Nicotiana tabacum* cells during the expression of cultivar-race and non-host resistance towards *Phytophthora* spp. (Perrone et al., 2003). However, it is less clear how ROS participate in compatible plant-pathogen interactions. $O^{\cdot}{}_2^-$ content increased in both lines before virus accumulation could be detected, and an increase in H_2O_2 was observed in the tolerant L33 in that period. Though the origin of such increases was not investigated, it is clear these changes in ROS production did not prevent virus systemic spread, in contrast to what happens in incompatible reactions. In L2, the increase in $O^{\cdot}{}_2^-$ was followed by increases in SOD and CAT activities; it is possible these enzymes interrupted signals generated by the increase in $O^{\cdot}{}_2^-$, which may have otherwise triggered defence reactions. Increases in the antioxidant enzymes were not observed in response to SuCMoV infection in the more tolerant L33 genotype, which showed slower virus accumulation along with an initially higher H_2O_2 concentration (Figure 4), rendering support to the association between high ROS production (such as in incompatible reactions) and less virus spread.

Table 1. Effect of 1 mM SA treatment on SOD, CAT and APX activities in healthy or SuCMoV-infected sunflower plants of lines L2 and L33. Samples were taken two days before (BS) or on the day of symptom appearance (SE). Results are means ± SE of five plants. Figures in parenthesis are percentage of non-treated plants (values in Figure 5)

Enzyme	Genotype	Sampling time	Healthy	Inoculated
SOD	L2	BS	34.39 ± 0.49 (62)	35.19 ± 3.91 (13)
		SE	64.84 ± 13.41 (101)	65.68 ± 11.35 (79)
	L33	BS	19.06 ± 0.51 (47)	23.91 ± 2.93 (51)
		SE	42.30 ± 2.32 (141)	27.46 ± 1.36 (91)
CAT	L2	BS	17.19 ± 2.97 (97)	8.90 ± 1.83 (26)
		SE	6.75 ± 1.86 (50)	2.89 ± 0.13 (21)
	L33	BS	10.44 ± 1.73 (87)	8.13 ± 0.61 (82)
		SE	Nd	Nd
APX	L2	BS	0.462 ± 0.031 (156)	0.444 ± 0.088 (154)
		SE	0.342 ± 0.066 (180)	0.399 ± 0.045 (173)
	L33	BS	0.356 ± 0.029 (105)	0.308 ± 0.031 (152)
		SE	0.166 ± 0.016 (97)	0.246 ± 0.032 (129)

Nd = not determined.

Antioxidant enzyme activities increase in diverse environmental stress situations (Mittler, 2002), a response related to ROS detoxification. In compatible plant-virus interactions both inductions (Fodor et al., 1997; Riedle- Bauer, 2000), and decreases (Clarke et al., 2002) of several antioxidant enzymes have been reported. During the interaction between viruses and plants that develop compatible reactions, there are different patterns of gene expression that can be described as early, transient and late responses (Havelda and Maule, 2000). In virus-infected peas, a gene encoding a cytoplasmic glutathione reductase has an early and transient induction pattern, (Komives et al., 1998), similar to the increases in SOD and CAT activities we have observed in our work. SuCMoV triggered an increase in antioxidant enzyme activities in L2; it was expected that treatments such as SA that decreased virus concentration (Murphy et al., 1999) would buffer the changes in antioxidant enzyme activity. In L2, 1 mM SA treatments that reduced virus concentration (Figure 8) were effective in preventing infection-associated increases in both SOD and CAT (Table 1), and stimulated H_2O_2 production (Figure 9). The results of the SA treatments also agree with the hypothesis that reduced virus concentration is a consequence of increased ROS production in the tissues, and provide support to the idea that the increase in antioxidant activity observed in L2 facilitates virus systematization. Therefore, in compatible interactions, the stimulation of the antioxidant defence may represent a virus-directed protection against host defences (Maule et al., 2002). Support for these interpretations requires information on the behaviour of the plant defences in this pathosystem, and such research is under way.

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