Oxidative stress induced by long-term Plum pox virus infection in peach (*Prunus persica* L. cv GF305)

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Abbreviations - ASC, ascorbate; AOS, activated oxygen species; ASC-GSH cycle, ascorbate-glutathione cycle; APX, ascorbate peroxidase; BSA, Bovine serum albumin; CAT, catalase; CO-protein, carbonil proteins contents; DHAR, dehydroascorbate reductase; EDTA, Ethylenediaminetetraacetic acid; F_v/F_m, maximum quantum yield of photosystem II; F'_v/F'_m, efficiency of excitation energy capture by PSII; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; 4-MN, 4-methoxy-1-naphthol; MOPS, 3-(N-morpholino)propanesulfonic acid, NPQ, non-photochemical quenching; $^1O_2$; singlet oxygen; $^{\cdot}OH$, hydroxyl radical; $O_2^{\cdot-}$, superoxide radical; pHMB, p-hydroxy mercury benzoic acid; PaMMoV; paprika mild mottle virus; PMMoV; pepper mild mottle virus; PPV, plum pox virus; PPV-D, Plum pox virus Dideron Type isolate; PVPP; Poly(vinylpolypyrrolidone); q_p; photochemical quenching; SOD superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TRSV, tobacco ringspots virus; Φ_{PSII}, quantum yield of photosystem II photochemistry.

Key Words: antioxidant enzymes; biochemical markers; oxidative stress; peach; PPV; *Prunus*; sharka.

In this study, the effect of long-term Plum pox virus (PPV) infection in the response of some antioxidant enzymes, at subcellular level, was studied in plants of peach (*Prunus persica* (L.) Batch) cultivar GF305, characterised by its great susceptibility to this virus. In infected plants, a decrease in the efficiency of excitation energy capture by PSII (F'_v/F'_m) was observed, that was accompanied by a decrease in the non-photochemical quenching (NPQ). p-hydroxy mercury benzoic acid-insensitive APX activity (class III peroxidase) was detected in both chloroplastic and soluble fractions. In soluble fractions from inoculated peaches, a significant increase in pHMB-sensitive APX and a significant decrease in SOD activity were observed. These changes were correlated with what occurred in isolated chloroplasts, where an increase in both pHMB-sensitive and pHMB-insensitive APX activities was observed, whereas a significant SOD, MDHAR and GR decreases were produced. According to these results, as a consequence of PPV infection, an oxidative stress, indicated by an increase in lipid peroxidation and in protein oxidation, was produced in peach leaves, which was monitored by the DAB-peroxidase coupled H_2O_2 probe. PPV infection produced an alteration in chloroplast ultrastructure, giving rise to dilated thylakoid membranes. PPV-infected peach leaves showed a lower amount of starch in chloroplasts from palisade parenchyma, as well as an increase in the number and size of plastoglobuli, in relation to control plants. The results suggest that long-term PPV infection produced an oxidative stress and an antioxidative metabolism imbalance may be related to the progress of PPV infection and symptoms in peach plants.

Introduction

Plum pox virus (PPV, sharka disease) is a serious limiting factor for temperate fruit production in those areas that are affected. PPV affects most *Prunus* species, resulting in severe economic losses in apricot, plum, prune and peach (Kölber 2001). In the short term, one way to control the spread of sharka in orchards is to remove infected trees and to use certified healthy plants. An alternative solution for eradication of the disease is the use of resistant cultivars (Dicenta et al. 1999). Obtaining *Prunus* cultivars resistant to sharka is one of the main objectives of breeders. The evaluation of programmes for PPV resistance is time-consuming and very expensive (Martínez-Gómez and Dicenta 2000a). Therefore, the search for biochemical and molecular markers associated with resistance would be of great interest. These markers will improve the selection process, in the evaluation of a higher number of individuals.

One of the most outstanding events in the early phase of incompatible plant-pathogen interaction is the rapid and transient production of activated oxygen species (AOS), such as $O_2^{\cdot -}$, H_2O_2 , $^{\cdot}OH$ and $^1O_2$, called the oxidative burst (Baker and Orlandi 1995). It is thought that a plasma membrane-associated NAD(P)H oxidase is activated during the response of plants to pathogens (Jabs et al. 1996). This results in the production of $O_2^{\cdot -}$, which dismutates, spontaneously or via superoxide dismutase (SOD; EC 1.15.1.1), into H_2O_2. However, H_2O_2 could also be produced enzymatically by peroxidase, urate oxidase, xantine oxidase or glucose oxidase (Bolwell et al. 1998, Halliwell and Gutteridge 1989, Montalbino 1992). Several studies have indicated that in incompatible reactions AOS are key mediators of programmed cell death during the hypersensitive response (Levine et al., 1994; Jabs et al., 1996). In contrast to incompatible reactions, little is known about the involvement of AOS in symptom development and pathogenesis in compatible plant-virus interactions.

Plants contain several mechanisms that detoxify $O_2^{\cdot -}$ and H_2O_2, called antioxidant systems. The primary components of these antioxidant systems include non-enzymatic antioxidants (carotenoids, ascorbate, glutathione and tocopherols) and enzymes such as SOD, catalase (EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), peroxidases and the enzymes involved in the ascorbate-glutathione cycle (ASC-GSH cycle); ascorbate peroxidase (APX, EC 1.11.1.1), dehydroascorbate reductase (DHAR, EC 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2). The components of this antioxidant defence system can be found in different subcellular compartments (Jiménez et al. 1998, Hernández et al. 2000).

An increasing body of data support the hypothesis that a fine regulation of antioxidant systems is part of the signalling pathways activating defence responses. However, the diversity in the systems used for studying plant-pathogen interplay make it difficult to formulate a clear picture of whether, and to what extent, changes in antioxidant systems are directly involved in the actuation of plants defence responses or are a mere consequence of the oxidative stress occurring in the attacked cells (de Gara et al., 2003). Several lines of evidences support the regulatory role that cellular antioxidants, especially GSH and GSH-related enzymes, play in biochemical and physiological responses of plants to biotic stress (Gullner et al., 1999; Fodor et al., 1997). In this sense, exposure of tobacco leaf discs to the cysteine precursor L-2-oxo-4-thiazolidine-carboxylic acid led to a massive accumulation of GSH as well as in a reduced TMV coat protein contents and suppression of disease symptoms in TMV-inoculated tobacco plants (Gullner et al., 1999). In a recent paper, it has been proposed that a decline in AOS scavenging capacity may be required before a rapid increase in virus replication could take place. *Phaseolus vulgaris* L. plants treated with the cytokinin dihidrozeatin, salicylic acid or

jasmonic acid showed elevated CAT, GR and peroxidase activities. These treatments, when applied before inoculation with White clover mosaic potexvirus, inhibited virus replication and symptom development (Clarke et al., 2002).

Most of the studies with PPV have been carried out at the level of characterisation of nucleotide sequence, identification of the pathogenicity determinants, characterization of hybrid potyviruses at the morphological level, studying their replication and mapping for PPV resistance (Martin and García 1991, Sáenz et al. 2000, Tóbiás et al. 2001, Hurtado et al. 2002). However, no data concerning the response of antioxidant systems to PPV infection at the subcellular level have been reported for woody plants.

In this study, the effect of PPV infection on fluorescence parameters and the response of some antioxidant enzymes were studied in soluble fractions and chloroplasts from peach leaves cv. GF305, characterised by its great susceptibility to sharka disease. The extent of lipid peroxidation, protein oxidation, the histochemical detection of H_2O_2 and the leaf ultrastructure were also analysed, in order to know if oxidative stress could be involved in the symptoms development and pathogenesis produced by this virus in peach leaves.

Material and Methods

Plant Material

Seedlings of peach (*Prunus persica* L.) rootstock GF305, characterised by their susceptibility to fruit viruses including PPV (Bernhard et al. 1969) and usually used as a rootstock in PPV resistance tests on *Prunus,* both *in vivo* (Martínez-Gómez and Dicenta 2000a) and *in vitro* (Martínez-Gómez and Dicenta 2000b), were assayed. GF305 peach seedlings were grown in 2-litre pots in an insect-proof greenhouse. Five repetitions of three-month-old seedlings were inoculated by grafting a chip from an

herbaceous GF305 individual showing strong sharka symptoms. Another five repetitions were kept as a control. Two months after inoculation, seedlings were subjected to an artificial rest period, in a cold chamber at 7 °C, in darkness for six weeks. They were then transferred to the greenhouse and were inspected for sharka symptoms 4 weeks later. In addition, to ascertain the presence or absence of Plum pox virus, an ELISA-DASI (detection of PPV coat protein) and an RT-PCR (detection of PPV nucleic acid) were applied to the leaves.

PPV isolate

The PPV isolate used was RB3.30, a Dideron Type isolate obtained from the Red Beaut plum cultivar in Spain, from the PPV collection of the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Valencia (Spain). This isolate is considered to be representative of the Spanish PPV population, and produces strong sharka symptoms in young leaves, consisting of venal chlorosis in peach GF305, and venal chlorosis and rings in susceptible apricot leaves (Pelet and Bovey 1968).

PPV detection

Symptoms in the GF305 leaves were scored on a scale from 0 (no symptoms) to 5 (maximum severity), as is usual in resistance tests in apricot (Martínez-Gómez and Dicenta 2000a). In addition, an ELISA-DASI (Double Antibody Sandwich Indirect) test was applied to the leaves, using the 5B monoclonal antibody against the coat protein of PPV (Cambra et al. 1994). Optical densities (OD) were recorded at 405 nm after 60 min. Samples with OD double that of the healthy control were considered as ELISA-positive (Sutula et al. 1986). Finally, for the detection of PPV nucleic acid, an RT-PCR analysis was carried out, using total RNA extracted using the Rneasy Plant Mini Kit (Qiagen, Hilden, Germany) as described by MacKenzie et al. (1997). Two specific

primers within the coat protein (CP) gene, VP337 (CTCTGTGTCCTCTTCTTGTG), complementary to positions 9487-9508 of genomic PPV, and VP338 (CAATAAAGCCATTGTTGGATC), homologous to 9194-9216 positions, were assayed. PCR parameters were: one cycle at 94 ºC for 2 min followed by 30 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec and 72 ºC for 30 sec, and finally an extension temperature of 72 ºC for 5 min (Martínez-Gómez et al. 2003). Amplified products were electrophoresed in 1% agarose gels in 40 mM Tris-acetate and 1 mM EDTA, pH 8.0, and stained with ethidium bromide.

Fluorescence measurements

Ten control and PPV-infected peach plants were analysed. Modulated chlorophyll fluorescence was measured in dark-adapted peach leaves at midday, using a chlorophyll fluorometer OS-30 (Optisciences, USA) with an excitation source intensity of 2000 µmol m^{-2} s^{-1}. The quantum yield of photosystem II photochemistry (Φ_{PSII}) was calculated empirically as the fluorescence parameter ($F_m' - F_t)/F_m'$ (Genty et al. 1989), and the maximum quantum yield of photosystem II (F_v/F_m) as ($F_m - F_o)/F_m$ (Maxwell and Johnson 2000). Non-photochemical quenching (NPQ) was calculated as a Stern-Vollmer-type quenching (Bilger and Björkman 1990). The photochemical quenching coefficient, equivalent to the fraction of open PSII reaction centres, was calculated as $q_p= (F'_m-F_t)/(F'_m-F'_o)$ (Maxwell and Johnson 2000).

The efficiency of excitation energy capture by PSII, corresponding to the probability that an absorbed photon reaches the PSII reaction centres, was calculated in light-adapted leaves as $F'_v/F'_m= (F'_m-F'_o)/F'_m$.

The minimal “dark” fluorescence level following illumination (F_o') was measured in the presence of a background far-red light, to favour rapid oxidation of intersystem electron carriers.

Isolation of cell fractions

For the isolation of cell fractions four weeks-old plants were used. All operations were carried out at 0-4 ºC. Soluble fractions were prepared by homogenising 3 g of fresh leaf material with a mortar and pestle, with 6 ml of a grinding medium containing 0.35 M mannitol, 30 mM MOPS buffer (pH 7.5), 4 mM L-cysteine, 1 mM EDTA, 5% insoluble PVPP (w/v) and 0.2% (w/v) BSA. For APX activity, 20 mM ascorbate was added. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 2200 g for 30 s, to pellet the chloroplast fraction. The supernatant was centrifuged at 12000 g, to discard mitochondria and peroxisomes. Then, the 12000 g supernatant was centrifuged for 20 min at 82000 g. The resulting supernatant obtained was partially purified, in Sephadex G-25 NAP columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with the same buffer (with or without 2 mM ascorbate) used for homogenisation, and was considered as the soluble fraction for use in different assays.

Chloroplasts were prepared by homogenising 5 g, of fresh leaf material, with a mortar and pestle, with 15 ml of a grinding medium containing 0.35 M mannitol, 30 mM MOPS buffer (pH 7.5), 4 mM L-cysteine, 1 mM EDTA, 5% soluble PVP (w/v) and 0.2% (w/v) BSA. For APX activity, 20 mM ascorbate was added. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 2200 g for 30 s; the resulting pellet was suspended in 0.3 M mannitol, 20 mM MOPS buffer (pH 7.0), 1 mM EDTA and 0.2% BSA (washing medium), with or without 2 mM ascorbate. The suspension was centrifuged at 2200 g for 30 s, and the pellet obtained was resuspended in 6 ml of the same washing medium. Resuspension medium containing 40% (v/v) Percoll (Amersham Pharmacia Biotech) was layered under the chloroplasts suspension by slowly pipetting 5 ml into the bottom of the tube. Tubes were centrifuged at 1700 g for 1

min. The pellet of intact chloroplasts was resuspended in 1 ml of washing medium, without BSA, and used for enzyme assays.

Assays performed

Catalase and the ASC-GSH cycle enzymes were measured as described in (Hernández et al. 2001a, 2001b). SOD activity was assayed by the ferricytochrome c method using xanthine/xanthine oxidase as the source of $O_2^{\cdot -}$ radicals (McCord and Fridovich, 1969). Total peroxidase was analysed according to (Ros-Barceló 1998). The extent of lipid peroxidation in leaves was estimated by determining the concentration of substances reacting with thiobarbituric acid (TBARS) (Cakmak and Horst 1991). Protein oxidation(CO-protein contents) was measured by reaction with 2,4-dinitrophenylhydrazine as described by Levine et al. (1990).

Histochemical detection of H_2O_2 in peach leaves

The histochemical detection of H_2O_2 in peach leaves was performed using endogenous peroxidase-dependent *in situ* histochemical staining, in which whole leaves were vacuum-infiltrated with 0.1 mg ml^{-1} 3,3'-diaminobenzidine in 50 mM Tris-acetate buffer (pH 5.0) and incubated at 25 ºC, in the dark, for 24 h. Controls were performed in the presence of 10 mM ascorbic acid. Leaves were rinsed in 80 % (v/v) ethanol for 10 min at 70 ºC, mounted in lactic acid:phenol:water (1:1:1, v/v/v) and photographed directly using an Olympus SZX 12 microscope (Hernández et al. 2001b).

Transmission electron microscopy

For microscopy, samples were fixed for 2.5 h, at 4 ^{o}C, in a 0.1 M Na-phosphate buffered (pH 7.2) mixture of 2.5 % glutaraldehyde and 4 % paraformaldehyde (Morales et al. 2001). Tissue was postfixed with 1% osmium tetroxide, for 2 h. The

samples were then dehydrated in a graded alcohol series and embedded in Spurr´s resin. Blocks were sectioned on a Reichert ultramicrotome (Germany). Thin sections for transmission electron microscopy were placed on copper grids and stained with uranyl acetate, followed by lead citrate. The ultrastructure of the tissue was observed with a Philip Tecnai electron microscope (Germany).

Results

GF305 peach proved to be very susceptible to the PPV-D isolate assayed in our controlled inoculation conditions. The evaluated repetitions developed strong PPV symptoms in leaves. The mean intensity of PPV symptoms of all the infected repetitions was around 3.0, in a scale from 0 to 5, confirming the high susceptibility described in this cultivar, and their use as PPV-indicator plants. In addition, PPV presence in leaves was confirmed by an ELISA-DASI test (presence of PPV coat protein) and RT-PCR analysis (presence of PPV nucleic acid), both positives (Table 1, Fig. 1). These serological and molecular techniques also confirmed the high PPV susceptibility of GF305 peach cultivar.

In infected plants, no evident changes in the PSII efficiency (F_v/F_m), the quantum yield of PSII electron transport (Φ_{PSII}) or the photochemical quenching coefficient (q_p) was produced. However, a decrease in the efficiency of excitation energy capture by PSII (F'_v/F'_m) was observed. This decrease in F'_v/F'_m was accompanied by a decrease in the non-photochemical quenching (NPQ) (Table 2), that could results in a diminished capacity of thermal dissipation of excess excitation energy in PSII.

In GF305 peach, p-hydroxy mercury benzoic acid-insensitive APX activity (class III peroxidase) was detected in both chloroplastic and soluble fractions. These peroxidases exhibit high specific APX activity and oxidise ASC and organic phenols

at comparable rates (Kvaratskhelia et al. 1999). Specific inhibitors of APX, such as p-chloro-mercury benzoate, hydroxy urea, p-aminophenol or p-hydroxy-mercury benzoate (pHMB), have only a slight effect on the ASC-dependent peroxidase activity of this type of peroxidases.

In soluble fractions from the inoculated peaches, long-term PPV infection produced a significant increase in pHMB-sensitive APX, whereas a significant decrease in soluble SOD activity was observed. However, no changes in the other antioxidant enzymes studied (CAT, MDHAR, DHAR, GR, pHMB-insensitive APX or total peroxidase) were produced in the soluble fraction from peach leaves in response to PPV (Table 3).

Long-term PPV infection also produced an alteration in the levels of antioxidant enzymes in leaf chloroplasts from peach plants cv GF305. In these organelles, significant increases in both pHMB-sensitive and pHMB-insensitive APX activities were observed. However, a significant decrease in chloroplastic SOD, MDHAR and GR occurred in response to the infection, whereas no changes in chloroplastic DHAR were observed (Table 4). According to these data, it seems that chloroplasts were more affected to PPV infection than the cytosolic compartment.

As a consequence of long-term PPV infection, an oxidative stress was produced in peach leaves, as reflected by the 70 % increase in lipid peroxidation (given as TBARS) and the 2.27-fold increase in protein oxidation (measured as CO-proteins) (Fig 2). In younger leaves (2-weeks-old leaves), an increase in lipid peroxidation was observed also, although values were lower than that observed in older leaves (1.684 ± 0.013 and 2.360 ± 0.062 nmol g^{-1}FW, for control and PPV-infected leaves, respectively).

When long-term PPV-infected leaves were stained with DAB reagent, to locate H_2O_2 production, a red-brown staining was observed in mesophyll cells near the minor veins (Fig. 3A, C). This staining seemed to be due to H_2O_2, since it was totally suppressed by 10 mM ascorbic acid and no staining was observed in control plants (Fig. 3B,D) or in asymptomatic infected leaves (not shown). The DAB-staining was even more marked in leaves after two weeks of infection (Fig. 3A).

Control plants showed a well-developed ultrastructure, mitochondria, endoplasmatic reticulum, golgi and nucleus. Chloroplasts from control plants exhibited high numbers of starch granules per chloroplast and only showed a low number of plastoglobuli (Fig. 4A, B). However, PPV-infected peach leaves showed a lower amount of starch in chloroplasts from palisade parenchyma, as well as an increase in the number and size of plastoglobuli (Fig. 4C-D-E). PPV infection produced mainly an alteration in chloroplast ultrastructure, giving rise to dilated thylakoid membranes (Fig. 4F), but other organelles were unaffected (Fig. 4E-F).

Discussion

The peach cultivar GF305 is very susceptible to PPV, as shown by the strong chlorosis symptoms observed after 4 weeks of infection and as described previously (Martínez-Gómez and Dicenta 2000a, 2000b). Mean intensity of PPV symptoms range normally around 1 to 2 in the ligneous hosts as the *Prunus* species (Martínez-Gómez and Dicenta, 2000a). In addition, optical densities of ELISA were very high in comparison with date observed in other peach cultivars (Cambra et al. 1994).

PPV infection produced a decrease in F'_v/F'_m and NPQ. The decrease in F'_v/F'_m has been described also in TMV-infected tobacco plants and in PMMoV- and PaMMoV-infected *Nicotiana bentamiana* plants (Van Kooten et al. 1990, Rahoutei et al. 2000), indicating a lower efficiency of excitation energy capture by open PSII reaction centres. The reduction in F'_v/F'_m could be attributed also, in part, to the

destruction of PSII reaction centres (Rahoutei et al. 2000). However, this was not the case for peach plants, because the values for F_v/F_m were around 0.8 for both control and PPV-infected plants. In virus-infected tobacco and *Nicotiana bentamiana* plants, the decrease in F'_v/F'_m was parallel with an increase in NPQ (Van Kooten et al. 1990; Rahoutei et al., 2000). The maintenance of NPQ values under stress situations has been associated with a capacity to dissipate light energy safely, and it can be seen as a protective response in order to avoid photoinhibitory damage to the reaction centres (Van Kooten et al. 1990, Rahoutei et al. 2000). However, a decrease in NPQ was observed in PPV-infected peach leaves, that could reflect a diminished capacity for the safe dissipation of excess light energy, and therefore does not avoid the production of harmful species, such as $^1O_2$ (Fryer et al., 2002).

In most incompatible responses, the rapid induction of highly localised events determines unfavourable conditions for pathogen growth. This defence response culminates in a localised cell death, called the hypersensitive response, designed to impair pathogen spread (De Gara et al. 2003). It has been suggested that during the first hours of barley-mildew interaction, H_2O_2 could contribute to the first line of defence against mildew invasion (Thordal-Cristensen et al. 1997). However, in the present work, different factors, including the use of woody plants, the mode of inoculation (a piece of bark from diseased GF305 plants) and the time which passed between the subjection of plants to the artificial dormancy and the growth of the first expanding leaves, made it difficult to study the short-term responses against PPV infection, and results were obtained for long-term PPV infection. Changes in antioxidant metabolism have also been reported in compatible plant-virus interactions, but contradictory results were shown, with both induction and reduction of antioxidant enzymes activities being described (Riedle-Bauer 2000, Hernández et al. 2001a, Clarke et al. 2002). These studies suggested that AOS could act as signal

activating defence genes and that regulation of antioxidant enzymes could be important in determining susceptibility or resistance, indicating that AOS and antioxidant metabolism could be involved in the symptom development and pathogenesis (Riedle-Bauer 2000; Hernández et al. 2001a, Clarke et al. 2002; de Gara et al., 2003).

It has been described that ASC oxidation may also be catalysed by pHMB-insensitive class III peroxidases (Class III family) (Kvaratskhelia et al. 1999). They exhibit high specific APX activity and oxidise ASC and organic phenols at comparable rates (Kvaratskhelia et al. 1999). In leaves from GF305 peach, pHMB-insensitive APX activity has been detected in both chloroplasts and soluble fractions. The increases in soluble and chloroplastic pHMB-sensitive APX, as well the increase in chloroplastic pHMB-insensitive APX and the maintenance of soluble catalase, pHMB-insensitive APX and total peroxidase activities, could have prevented an excessive H_2O_2 accumulation in leaves, and thus may have led to a reduced signal transduction effect for defence genes induction (Levine et al. 1994). Alternatively, if these H_2O_2-scavenging enzymes, were important for resistance against PPV, they may have occurred too late to afford protection against the oxidative stress induced by the long-term PPV infection. On the other hand, the increases in APX activities observed in peach leaves could be also a response to cellular damage, rather than a direct response to the presence of PPV. Probably, this increase in APX activity could not stop the development of symptoms that resulted from the infection by PPV, but could have helped to reduce the severity of the disease and, probably, to allow the plants to recover. This situation is similar to that described in leaves of *Dactylis glomerata* L., susceptible to Cocksfoot mottle virus, where an increase in antioxidant enzymes was described in response to the cellular damage imposed by long-term virus infection (Li and Burritt 2002).

No changes could be observed in soluble DHAR or MDHAR activities from infected plants. However, values were higher for DHAR than for MDHAR (nearly 2-fold), suggesting that, in cytosol from peach leaves, ASC could be regenerated mostly *via* DHAR. Conversely, in chloroplasts from control leaves, MDHAR values were higher than these of DHAR, suggesting that ASC could be regenerated mostly *via* MDHAR. However, in infected plants, decreases in chloroplastic MDHAR and GR occurred, suggesting that these plants could have a lower capacity for regeneration of chloroplastic ASC and GSH. This, linked to the observed decreases in soluble and chloroplastic SOD, could favour the long-term PPV-induced oxidative stress, as shown by the increases in lipid peroxidation and protein oxidation and the appearance of oxidative microbursts. In two apricot cultivars, foliar DHAR activity increased in response to PPV infection, but the rise was much higher in the resistant plants (300%) that in the susceptible ones (only 37%), suggesting that the inoculated resistant cultivar had a higher capacity for regeneration of ASC than the inoculated susceptible plants (Hernández et al. 2001a). This higher DHAR induction in the PPV-resistant cultivar could contribute to an increased antioxidant capacity, which could be related, among other factors, to their resistance to PPV. Besides ASC, it has been reported that GSH can be also important in the reduction of both, number of necrotic lesion and virus contents in TMV-infected tobacco plants (Gullner et al., 1999).

In long-term PPV-infected leaves, H_2O_2 production has been observed in mesophyll cells near the minor veins. Such production was more intense in younger leaves and resembled the leaf microbursts observed by other authors in response to pathogenic stress situations where H_2O_2 is accumulated (Alvarez et al. 1998, Orozco-Cardenas and Ryan 1999). Similar microbursts has been observed in ozone-treated tobacco plants as well as in salt-treated pea plants, and in both cases, the appearance of DAB-stainable areas was in parallel with the establishment of an

oxidative stress (Schraudner et al. 1998, Hernández et al. 2001b). The accumulation of H_2O_2, despite the decreases in soluble and chloroplastic SOD, indicates that this metabolite could be produced also in other ways (by NADPH oxidase, peroxidase, xanthine oxidase, urate oxidase, glycolate oxidase or glucose oxidase) (Halliwell and Gutteridge 1989, Montalbino 1992, Bolwell et al. 1998). Therefore, the H_2O_2 production could be associated with the establishment of an oxidative stress caused by the long-term PPV infection. On the other hand, PPV infection produced intervenal chlorosis symptoms, which could have been induced by an increased generation of AOS, as shown in pea plants subjected to NaCl stress (Hernández et al. 2001b). As mentioned earlier, the decrease in NPQ in infected plants could increase the production of AOS in chloroplasts (Fryer et al. 2002), and it was accompanied by a decrease of chloroplastic antioxidant enzymes. Thus, the chlorosis symptoms observed in PPV-infected leaves could be ascribed both to a higher AOS generation and to a lower capacity for scavenging AOS.

Lipid peroxidation and protein oxidation are the symptoms most easily ascribed to oxidative damage and they are often used as indicator of oxidative damage (Gómez et al., 1999; Hernández et al., 2001b, 2004). In salt-treated plants, the observed increases in lipid peroxidation and protein oxidation were correlated with the rise in AOS production (Gómez et al., 1999; Hernández et al., 2001b). In TRSV-infected cowpea laves, AOS generation, in particular $O_2^{\cdot -}$, is responsible for the increased lipid peroxidation which resulted in membrane damage (Beleid El-Moshanty et al., 1993). In PPV-infected peach leaves, a decrease in both chloroplastic and soluble SOD took place. This could reduce the ability to eliminate $O_2^{\cdot -}$, thus increasing the risk of $^{\cdot}OH$ formation (Halliwell and Gutteridge 1989) that could contribute to the increases in lipid peroxidation and protein oxidation observed.

PPV infection produced some ultrastructural alterations in peach cv. GF305. The presence of dilated thylakoids and the increase in plastoglobuli seem to be a general stress response, because they have been described previously, both under biotic and abiotic stress situations. In *Chenopodium guinoa* plants infected with Saguaro cactus virus, chloroplasts were severely damaged, showing frequent dilations of lamellae, a lower number of grana and numerous large plastoglobuli (Russo and Marterlli 1982). Similarly, ultrastructural changes induced by Zucchini yellow mosaic virus in leaves of Styrian pumpkin plants revealed that, in chloroplasts of infected leaves, the amounts of plastoglobuli increased significantly, whereas the amount of thylakoids significantly decreased (Zechmann et al. 2003). The alterations induced in the chloroplast ultrastructure by pathogen attacks are similar to those induced by NaCl stress (Hernández et al. 1995; Morales et al. 2001). The lower amount of starch in chloroplasts from PPV-infected leaves could have been due to an increased demand of plant tissues for normal respiration and growth, caused by activated defence responses and by the requirements of the pathogen. Thus, competition among plant sinks and with the pathogen is increased, with the consequence that, in compatible interactions, less carbon from the nutrient pool is available for storage and translocation (Ayres et al. 1996).

Replication of some mammalian viruses is triggered by AOS and it is thought that cellular antioxidants may play an important role in preventing viral diseases (Schwarz 1996). In cell culture systems, H_2O_2 promotes replication of HIV while antioxidants, such as N-acetyl-cysteine or ASC, have the opposite effect (Harakeh et al. 1990, Staal et al. 1990). It has been proposed that a decline in free radical-scavenging capacity may be required before a rapid increase in virus replication can take place, and treatments increasing the ability of plants to scavenge AOS may hinder virus replication (Clarke et al., 2002). If the same situation occurs in PPV-

infected peach plants, the oxidative stress, accompanied by decreases in cytosolic and chloroplastic antioxidative enzymes, could be related to the progress of PPV infection and symptoms in leaves.

The results indicate that infection by PPV led to changes in the antioxidative enzymes of peach, both in the cytosol and in the chloroplasts. In conclusion, long-term PPV infection produced an oxidative stress in leaves of peach cv. GF305, characterised by its great susceptibility to sharka disease, as shown by the decreases in soluble and chloroplastic SOD and chloroplastic MDHAR and GR, the increases in lipid peroxidation and protein oxidation and the appearance of oxidative microbursts. This suggests that an antioxidative metabolism imbalance may be related to the progress of PPV infection and symptoms in peach leaves.

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Table 1. PPV detection in inoculated and control GF305 peach seedlings. Number of plants giving positive results by ELISA and/or RT-PCR analysis. The results of two different cycles of experiments are shown.

	Evaluated plants	Cycle 1			Cycle 2		
		Symptoms[a]	ELISA[b]	RT-PCR	Symptoms[a]	ELISA[b]	RT-PCR
Control	5	0 (0.0)	0 (0.07)	0	0 (0.0)	0 (0.06)	0
Inoculated	5	5 (3.5)	5 (2.40)	5	5 (3.0)	0 (2.26)	5

[a]Symptoms intensity on a scale from 0 (no symptoms) to 5 (maximum intensity).

[b]Mean optical density (OD) of ELISA at 405 nm after 60 minutes.

Table 2. Fluorescence parameters measured in control and long-term PPV-infected GF305 peach leaves.

Fluorescence parameters	Control plants	PPV-infected plants
F_v/F_m	0.803 ±0.010	0.795±0.005
F'_v/F'_m	0.781±0.040	0.662±0.021a
Φ_{PSII}	0.102±0.008	0.107±0.018
q_p	0.131±0.020	0.162±0.015
NPQ	0.300±0.008	0.252±0.017a

Data represent the mean ± SD from ten repetitions. Differences from control values are significant at $P<0.05$ (a), according to Duncan's multiple range test

Table 3. Effects of long-term PPV infection on antioxidant enzymes in soluble fractions from GF305 peach leaves.

Enzymatic activity	Control plants	PPV-infected plants
pHMB-sensitive ascorbate peroxidase nmol oxidised asc min^{-1} mg^{-1} protein	143.9±3.9	173.1±5.2a
pHMB-insensitive ascorbate peroxidase nmol oxidised asc min^{-1} mg^{-1} protein	28.2±2.5	30.7±1.8
Monodehydro ascorbate reductase (MDHAR) nmol oxidised NADH min^{-1} mg^{-1} protein	33.9±3.3	33.7±2.0
Dehydroascorbate reductase (DHAR) nmol reduced asc min^{-1} mg^{-1} protein	71.0±2.5	72.8±3.7
Glutathione reductase (GR) nmol oxidised NADPH min^{-1} mg^{-1} protein	25.3±2.0	28.4±1.5
Superoxide dismutase (SOD) U mg^{-1} protein	19.5±0.6	13.5±1.3a
Catalase µmol reduced H_2O_2 min^{-1} mg^{-1} protein	8.9±0.8	11±0.9
Peroxidase µmol oxidised 4-MN min^{-1} mg^{-1} protein	382.7±18.9	403.6±5.5

Data represent the mean ± SD from at least three repetitions. Differences from control values were significant at $P<0.05$ (a), according to Duncan's multiple range test.

Table 4. Effects of long-term PPV infection on antioxidant enzymes in chloroplast suspensions from GF305 peach leaves.

Enzymatic activity	Control plants	PPV-infected plants
pHMB-sensitive ascorbate peroxidase nmol oxidised asc min^{-1} mg^{-1} protein	205.0 ±19.2	1753.2 ±15.4b
pHMB-insensitive ascorbate peroxidase nmol oxidised asc min^{-1} mg^{-1} protein	162.0±24.0	289.1±14.5a
Monodehydro ascorbate reductase (MDHAR) nmol oxidised NADH min^{-1} mg^{-1} protein	99.84±9.94	36.83±2.14b
Dehydroascorbate reductase (DHAR) nmol reduced asc min^{-1} mg^{-1} protein	23.5±0.7	22.4±3.7
Glutathione reductase (GR) nmol oxidised NADPH min^{-1} mg^{-1} protein	27.7±2.5	20.1±1.5a
Superoxide dismutase (SOD) U mg^{-1} protein	19.2±0.6	14.0±1.2a

Data represent the mean ± SD from at least three repetitions. Differences from control values were significant at $P<0.05$ (a), $P< 0.01$ (b), according to Duncan's multiple range test.

Legend to Figures

Fig. 1. Amplification products (313 bp) indicative of the presence of PPV, obtained using RT-PCR for PPV detection in different samples. Lane 1: healthy GF305 seedling, Lane 2: GF305 seedling infected by PPV and showing strong sharka symptoms. M: molecular weight marker 1 Kb (Gibco BRL).

Fig. 2. Effect of long-term PPV-infection on lipid peroxidation (given as TBARS) and protein oxidation in GF305 peach leaves. Data represent the mean ± standard errors of at least three replicates. Differences from control values were significant at $P<0.05$ (a) or $P<0.01$ (b), according to Duncan's multiple range test. GFc, control plants; Gfi, PPV-inoculated plants.

Fig. 3. Detection of H_2O_2 generation in leaves from DAB-stained GF305 peach plants. A) two-week-old PPV-infected plants; B) two-week-old control plants; C) four-week-old PPV-infected plants; D) four-weeks-old control plants. Bars = 500 μm.

Fig. 4. Electron micrographs from GF305 peach leaves. A) Palisade parenchyma cells from control plants. B) Control plant. Detail of a chloroplast of a palisade parenchyma cell, showing large starch granules and few plastoglobuli. C) Palisade parenchyma cells from PPV-inoculated plants. D), E) Inoculated plants. Detail of chloroplasts of palisade parenchyma cells, showing few starch granules and a large amount of plastoglobuli. F) Inoculated plant. Detail of chloroplasts, showing dilated thylakoids and a large amount of plastoglobuli.

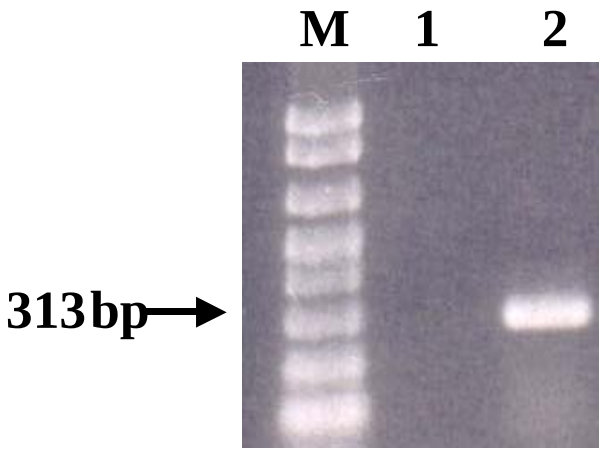
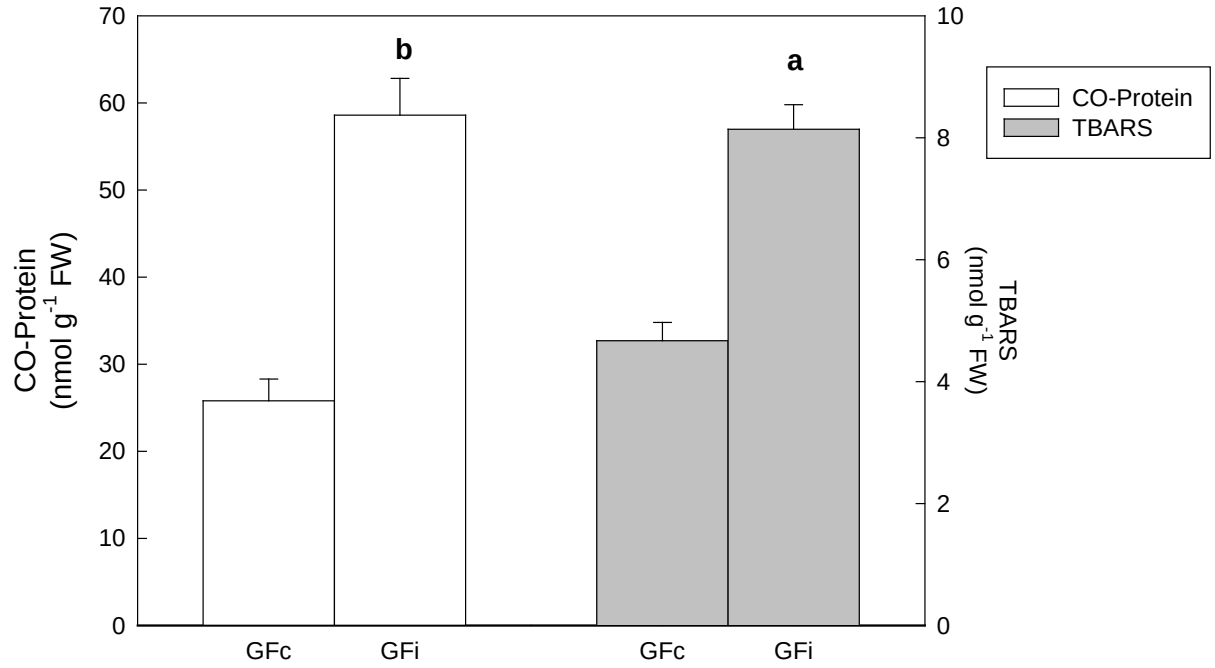


Fig. 1.


70
60
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CO-Protein
(nmol g-1 FW)
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8
6
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CO-Protein
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GFi
GFc
GFi

Fig. 2.

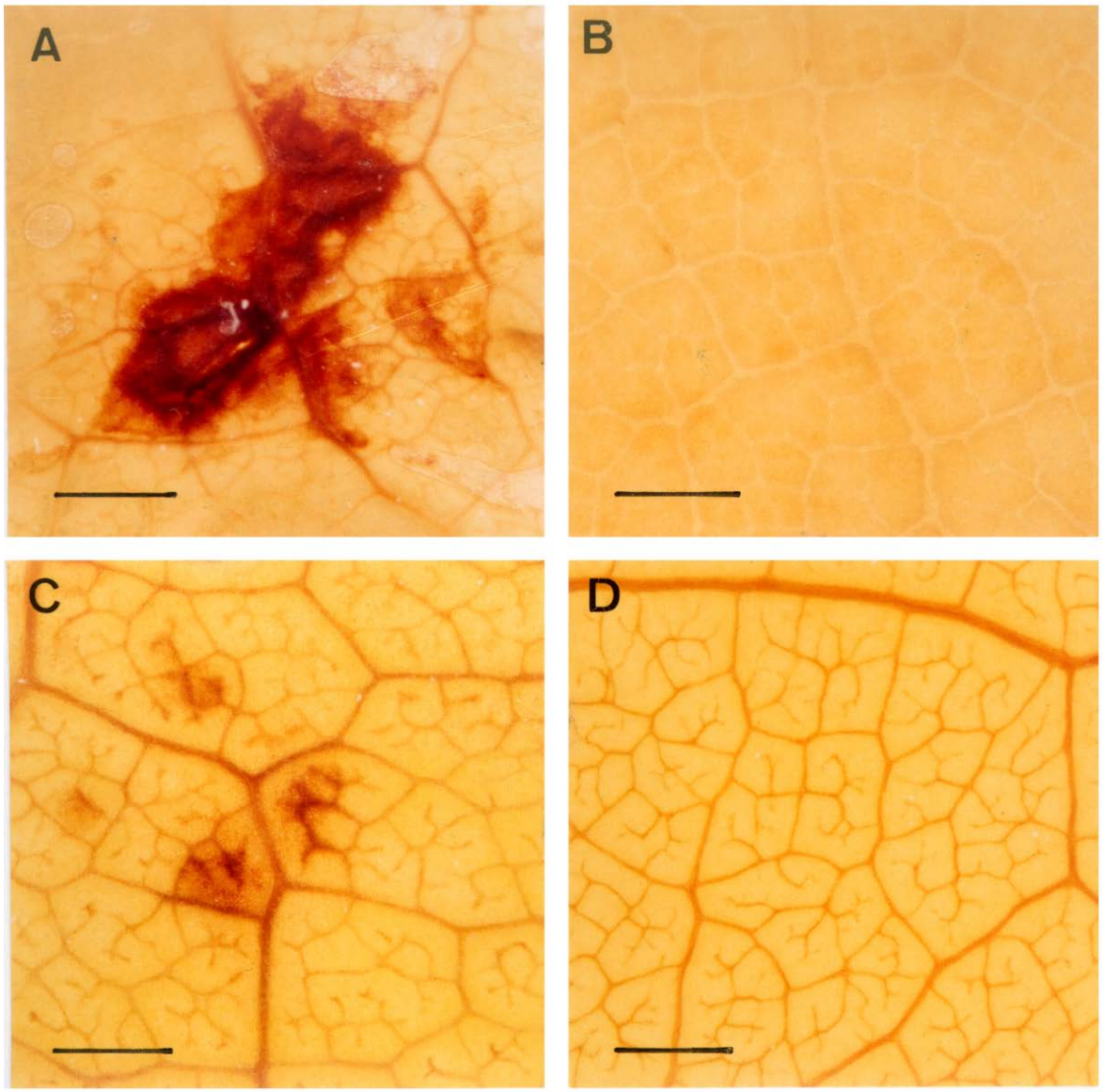

Fig. 3.

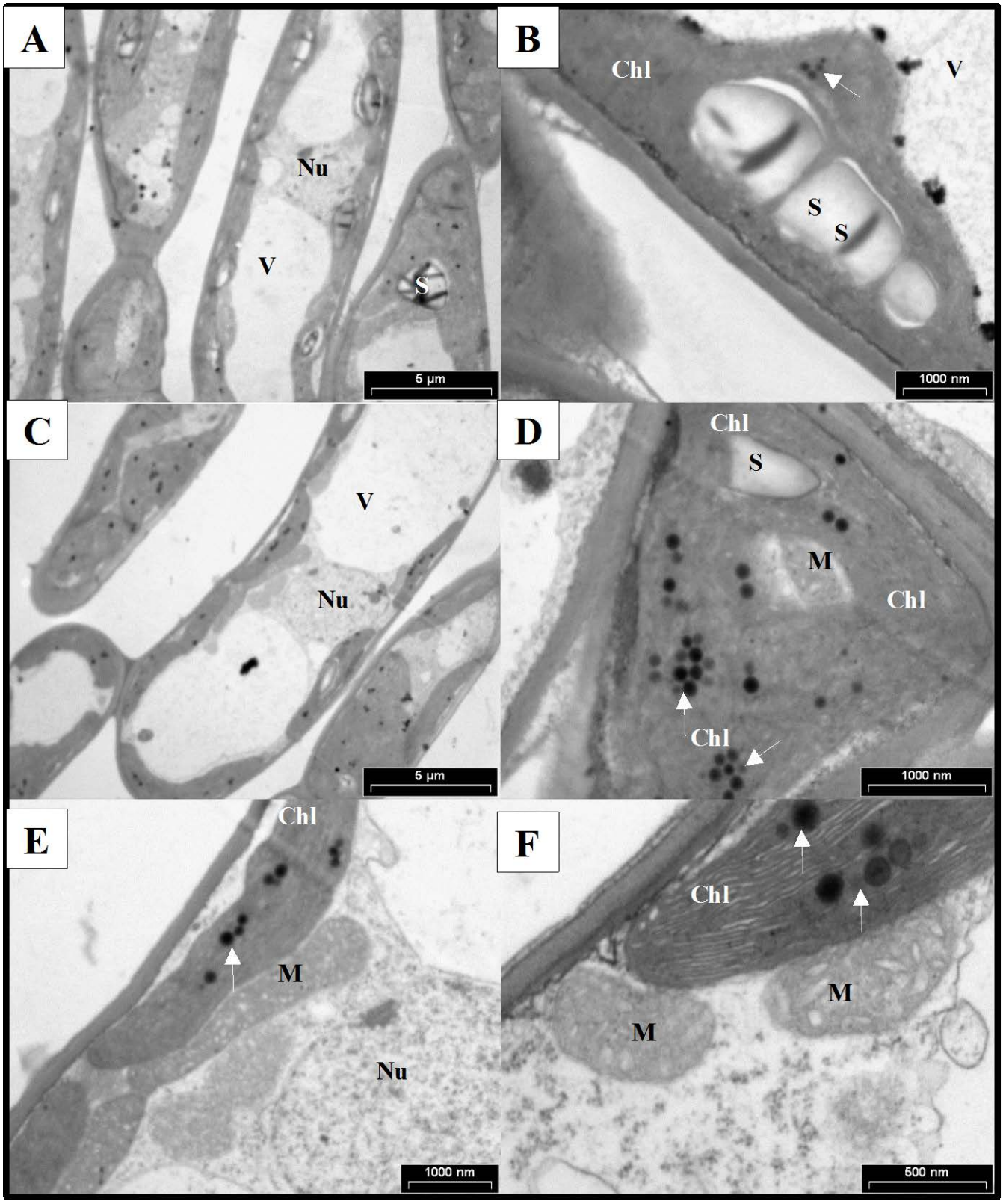
A
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Fig. 4.