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Hydrogen peroxide localization and antioxidant status in the recovery of apricot plants from European Stone Fruit Yellows

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Abstract

Hydrogen peroxide (H_2O_2) localization and roles of peroxidases, malondialdehyde and reduced glutathione were compared in leaves of apricot (*Prunus armeniaca*) plants: asymptomatic, European Stone Fruits Yellows (ESFY)-symptomatic and recovered. Nested PCR analysis revealed that '*Candidatus* Phytoplasma prunorum', is present in asymptomatic, symptomatic and recovered apricot trees, confirming previous observations on this species, in which recovery does not seem to be related to the disappearance of phytoplasma from the plant.

H_2O_2 was detected cytochemically by its reaction with cerium chloride, which produces electron-dense deposits of cerium perhydroxides. H_2O_2 was present in the plasmalemma of the phloem cells of recovered apricot plant leaves, but not in the asymptomatic or symptomatic material. Furthermore, by labelling apricot leaf tissues with diaminobenzidine DAB, no differences were found in the localization of peroxidases.

Protein content in asymptomatic, symptomatic and recovered leaves was not significantly different from one another. In contrast, guaiacol peroxidase activity had the following trend: symptomatic > recovered > asymptomatic, whereas reduced glutathione content followed the opposite trend: asymptomatic > recovered > symptomatic. Moreover, no differences were observed in malondialdehyde concentrations between asymptomatic, symptomatic and recovered leaves. The overall results suggest that H_2O_2 and related metabolites and enzymes appear to be involved in lessening both pathogen virulence and disease symptom expression in ESFY-infected apricot plants.

Introduction

Recovery, a spontaneous remission of symptoms in diseased plants, may or may not involve the elimination of the causal pathogen from the host (Schmid, 1965, 1975). Recovery has been reported to occur in apple, grape and apricot affected by phytoplasmas (Osler et al., 1993; 1999; Musetti et al., 2004).

In apple trees, recovery from apple proliferation (AP) correlates with the disappearance of phytoplasmas from the canopy but not from the roots (Seemüller et al., 1984; Loi et al., 2002). In the field, the probability of recovered apple plants becoming canopy-infected again is about four times lower than in plants not previously infected (Osler et al., 1999). In apricot plants affected by European Stone Fruit Yellows (ESFY), a disease caused by a phytoplasma belonging to the phylogenetic cluster 16SrX (Lee et al., 1998), recently proposed as '*Candidatus* Phytoplasma prunorum (*Ca.* Phytoplasma prunorum)' (Seemüller and Schneider, 2004), recovery does not appear to be correlated to the

disappearance of phytoplasmas from the plant (Osler et al., 1999).

Although recovery in apricot is not common, it is stable, thus suggesting the possibility of using cross-protected plants when planting apricot trees in ESFY infected areas (Morvan et al., 1986). Osler (unpublished) noticed that apricot plants obtained from recovered ones can be infected in nature, but to a lesser extent than never infected plants. These observations indicate that a type of systemic acquired resistance (SAR) is involved in inducing recovery (Ryals et al., 1992; Sticher et al., 1997).

It has been postulated that superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) play central, manifold roles in plant host as well as non-host resistance to pathogens (Baker and Orlandi, 1995). ROS-generating systems might kill pathogens and/or induce a hypersensitive response (HR), reinforce the plant cell wall or immobilize the pathogen within the cell, leading to the so-called incompatible plant pathogen reactions (Lamb and Dixon, 1997; Bowler and Fluhr, 2000).

In symptomatic, asymptomatic and recovered apple trees, differences in concentrations and activities of some ROS-related metabolites and enzymes, and H_2O_2 over-production in recovered plants were reported (Musetti et al., 2002), consequently, an active involvement of these compounds in the recovery phenomenon has been hypothesized (Musetti et al., 2004).

It has been proposed that several metabolic systems are involved in the scavenging of ROS, including the activation of oxidoreductase enzymes such as peroxidases (EC 1.11.1.7) (Bestwick et al., 1998; Vanacker et al., 2000), and the biosynthesis of metabolites such as reduced glutathione (GSH, γ-glutamyl-cysteinyl-glycine), which can be oxidized to glutathione disulfide (GSSG) in the presence of ROS (Vanacker et al., 2000). ROS, if not detoxified by the cellular antioxidant machinery, can cause lipid (membrane) peroxidation, with the consequent formation of numerous breakdown products, including malondialdehyde (MDA) (Esterbauer, 1982).

After the above mentioned studies on AP-infected apple plants (Musetti et al., 2002, 2004), we decided to change to ESFY-infected apricot plants because apple and apricot trees are two different biological systems, but, at the same time, involve two genetically closely related phytoplasmas (Lee et al., 1998).

Thus, the aims of this work were: (1) to verify if symptom remittance in recovered apricot plants is associated with the disappearance of '*Ca*. Phytoplasma prunorum' from the canopy; (2) to investigate, by cytochemical methods, the H_2O_2 and peroxidase localization in leaf tissues of recovered, symptomatic and asymptomatic apricot trees; (3) to study in the same material, by spectrophotometric and HPLC methods, variations in guaiacol peroxidase (GPX) activity, GSH content and MDA concentration possibly linked to the regulation of the recovery phenomenon.

Materials and methods

Plant material and phytoplasma detection

Twelve year-old apricot (*Prunus armeniaca)* trees (cv. Reale d'Imola, sensitive to ESFY) were grown in an organic orchard near Spessa, an area with a high natural rate of ESFY, in the Friuli-Venezia Giulia region (north-eastern Italy). Since 1991, for 12 consecutive years, field observations have been made to investigate the appearance and development of ESFY symptoms in apricot plants.

In the orchard, asymptomatic, symptomatic and recovered apricot plants were present at the time (years 2002 and 2003) of the experiments reported here. The asymptomatic apricot trees chosen for these experiments had never showed symptoms of ESFY since planting. On the contrary, symptomatic plants presented typical ESFY symptoms, such as yellows, leaf roll and reddening, premature leafing, abnormal flowering, severe phloem necrosis and death of some branches. Recovered trees, after an initial symptomatic period, remitted symptoms and showed regular growth and production.

For the investigations reported in this paper, samples were collected during September 2002 and 2003, when typical symptoms of the disease were evident.

The following apricot trees were chosen for analyses: five asymptomatic (since planting), five symptomatic (for at least four consecutive years) and five recovered (remained asymptomatic for at least four consecutive years after recovery).

No virus diseases were detected in the orchard. In particular, plum pox virus using ELISA, proved negative. The most common diseases caused by *Monilia* spp. and *Stigmina carpophila* were found to be uniformly distributed among symptomatic, asymptomatic and recovered plants.

In order to confirm the presence/absence of phytoplasmas in randomly sampled leaf tissues, during the same period, samples were tested by nested polymerase chain reaction (PCR).

DNA was extracted from 1.0 g of midveins using a phytoplasma enrichment procedure (Ahrens and Seemüller, 1992), and amplified using the universal primers P1/P7 (Schneider et al., 1995). After 1:40 dilution, 2 μl of PCR product obtained in the first amplification were used for further amplification with the nested primers f 01/r 01; 35 cycles of PCR were carried out under the following conditions: 30 s of denaturation at 95 °C, 75 s of annealing at 55 °C, and 90 s of extension at 72 °C (Lorenz et al., 1995). Amplification products were analysed by 1% agarose gel electrophoresis.

Hydrogen peroxide (H_2O_2) localization

The cytochemical method developed for the localization of hydrogen peroxide (H_2O_2) in plant tissues was used as described by Bestwick et al. (1995): small samples of leaves (1 × 3 mm) collected from asymptomatic, symptomatic and recovered apricot trees were incubated in freshly prepared 5 mM $CeCl_3$ in 50 mM 3-(N-morpholino)propanesulfonic acid at pH 7.2 for 1 h. Tissues were then fixed in 1.25% (vol/vol) glutaraldehyde/1.25% (vol/vol) paraformaldehyde in 0.1 M phosphate buffer pH 7.2 for 1 h. After fixing, samples were washed in buffer and dehydrated in ethanol, then embedded in Epon/Araldite resin. Several serial ultrathin sections of at least 80 samples from each group (asymptomatic, symptomatic and recovered plants) were stained with lead citrate and uranyl acetate, then observed under a Philips CM 10 transmission electron microscope (TEM) operating at 80 kV.

Peroxidase cytochemistry

Sexton and Hall's (1978) protocol, modified by Bestwick et al. (1998) was used. Small leaf samples (1 × 3 mm), collected from asymptomatic, symptomatic and recovered apricot trees, were fixed for 45 min at room temperature in a mixture of 1% (vol/vol) glutaraldehyde/1% (wt/vol) paraformaldehyde in 50 mM sodium phosphate at pH 7.0 (buffer A). Samples were then washed twice for 10 min in buffer A and transferred for 30 min into 50 mM potassium phosphate buffer at pH 7.8 (buffer B) or pH 6.0 (buffer C). After washing, samples were transferred to 0.5 mg ml^{-1} diaminobenzidine (DAB) and 5 mM H_2O_2 dissolved in either buffer B or C. To prevent autooxidation of DAB, the staining medium was freshly prepared and kept under a dim light. Control samples were incubated in a buffer B solution, without DAB.

Samples were incubated for 30 min in the dark, washed twice for 10 min in buffer B or C, postfixed in 1% osmium tetroxide for 1 h, then dehydrated in an ethanol series and embedded in Epon/Araldite resin. Several serial ultrathin sections of at least 80 samples (from asymptomatic, symptomatic and recovered plants, respectively) were stained with lead citrate and uranyl acetate, then observed under a PHILIPS CM 10 TEM operating at 80 kV.

Reduced glutathione (GSH) detection

Samples (200 mg for each extract, from asymptomatic, recovered and symptomatic leaves, respectively) were homogenised in a mortar, in ice-cold 5% (wt/vol) 5-sulfosalicylic acid, containing 6.3 mM diethylenetriaminepentaacetic acid, following De Knecht et al. (1994). After centrifugation at 10,000 × g for 10 min at 4 °C, the supernatant fraction of the extracts was filtered through a Minisart 0.45 μm filter (Sartorius, Germany) and immediately assayed by HPLC (model 200, Perkin Elmer, USA). GSH was separated through a reverse-phase C_{18} column (Purosphere, Merck, Germany), by injecting 200 μl of the filtered supernatant fraction. The GSH separation was obtained by using a 2% CH_3CN and 98% water isocratic flow, with the rate set at 0.7 ml min^{-1}. The elution solution contained 0.05% trifluoroacetic acid. GSH was determined using post-column derivatization with 300 μM Ellman's reagent [5,5′dithio(2-nitrobenzoic acid)], detected at 412 nm and measured by a calibration curve for standard SH groups. Identification of GSH was based on the comparison of its retention times with standard GSH (Merck, Germany).

Protein content, guaiacol peroxidase (GPX) activity and malondialdehyde (MDA) concentration assays

Samples (700 mg for each extract, from asymptomatic, recovered and symptomatic leaves) for protein content determination and GPX activity measurements were shock frozen in liquid nitrogen, homogenized on ice in the ratio of 1:4 (wt/vol) with 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM sodium ascorbate, 0.2 mM Na_2-EDTA, 1% (wt/vol) polyvinylpyrrolidone (PVP) and 1% (vol/vol) Triton X-100. Each homogenate, after gauze filtration, was centrifuged at $10{,}000 \times g$ for 25 min at 4 °C.

The resulting supernatants were subjected to protein concentration determination by Bio-Rad protein assay (Bio-Rad, Germany), using bovine serum albumin as a standard, mainly following Bradford (1976). GPX activity of supernatants was measured using a Varian Cary 1E spectrophotometer (Pandolfini et al., 1992), and expressed as ΔA_{470} min^{-1} mg^{-1} protein.

Samples (1 g for each extract, from asymptomatic, recovered and symptomatic leaves) for MDA analysis were homogenized in 0.1% (wt/vol) trichloroacetic acid, gauze-filtered and centrifuged at $10{,}000 \times g$ for 10 min. Supernatants were then treated with 0.5% 2-thiobarbituric acid in 20% trichloroacetic acid, in the ratio of 1:4 (vol/vol) (Heat and Packer, 1968). The mixture was incubated at 95 °C for 25 min, quickly cooled on ice and then centrifuged as above. MDA determination of supernatants was performed by a Varian Cary 1E spectrophotometer at (A_{532}–A_{600}), using an extinction coefficient of 155 mM cm^{-1}.

Statistics

Five asymptomatic, five symptomatic and five recovered trees were chosen for molecular, ultrastructural and biochemical analyses. All experiments were performed on at least three replicates using materials with similar general aspect, size and symptoms. The significance of the experimental results was evaluated, where appropriate, using the unpaired *t*-test (SigmaPlot 8.0). Data are expressed as mean ± SE of at least three independent measurements. Different letters in the figures indicate significant differences between treatments at $P \leq 0.05$.

Results

Phytoplasma detection, H_2O_2 and peroxidase localization

Nested PCR analysis performed in 2002 and 2003 revealed that '*Ca*. Phytoplasma prunorum' was always detected in leaves of asymptomatic, symptomatic and recovered apricot trees, thus confirming the pathogen presence in all the investigated material.

Ultrastructural analyses indicated similar results in both 2002 and 2003.

Phytoplasmas were observed in the phloem of leaves of all symptomatic plants (Figure 1a and b). The pathogen was not easy to detect, because the sieve tubes had often collapsed (Figure 1c), and a number were necrotic or contained phenolic substances (Figure 1d).

No deposits of electron-dense cerium perhydroxide (indicating the presence of H_2O_2) were observed in either symptomatic (Figure 1a–d) or asymptomatic apricot leaf tissues (Figure 2a). In the latter, cell morphology and organelles were well preserved, and phytoplasmas were not detected.

In recovered apricot leaves, no phytoplasmas were observed using TEM; some phloem cells had a reduced lumen, others were completely enclosed in dark material, but the cells were, in general, well preserved (data not shown). Unlike symptomatic or asymptomatic leaves, TEM observations of leaf tissues from recovered plants revealed a considerable accumulation of cerium perhydroxide (indicating the presence of H_2O_2) localized only in the plasmalemma of the sieve tube cells (Figure 2b–d). No precipitates were found in the companion cells and parenchyma tissue (data not shown).

Electron-dense staining, detecting the presence of peroxidase, was observed in xylem, phloem and in mesophyll tissue of apricot leaves; the precipitates were localized on the plasmalemma (data not shown). No differences in the localization of these electron-dense precipitates were revealed among symptomatic, asymptomatic and recovered plants and no electron-dense deposits were detected in the buffer-incubated control samples (data not shown).

Protein content and oxidative status

The protein content in asymptomatic, symptomatic and recovered apricot leaves was not significantly

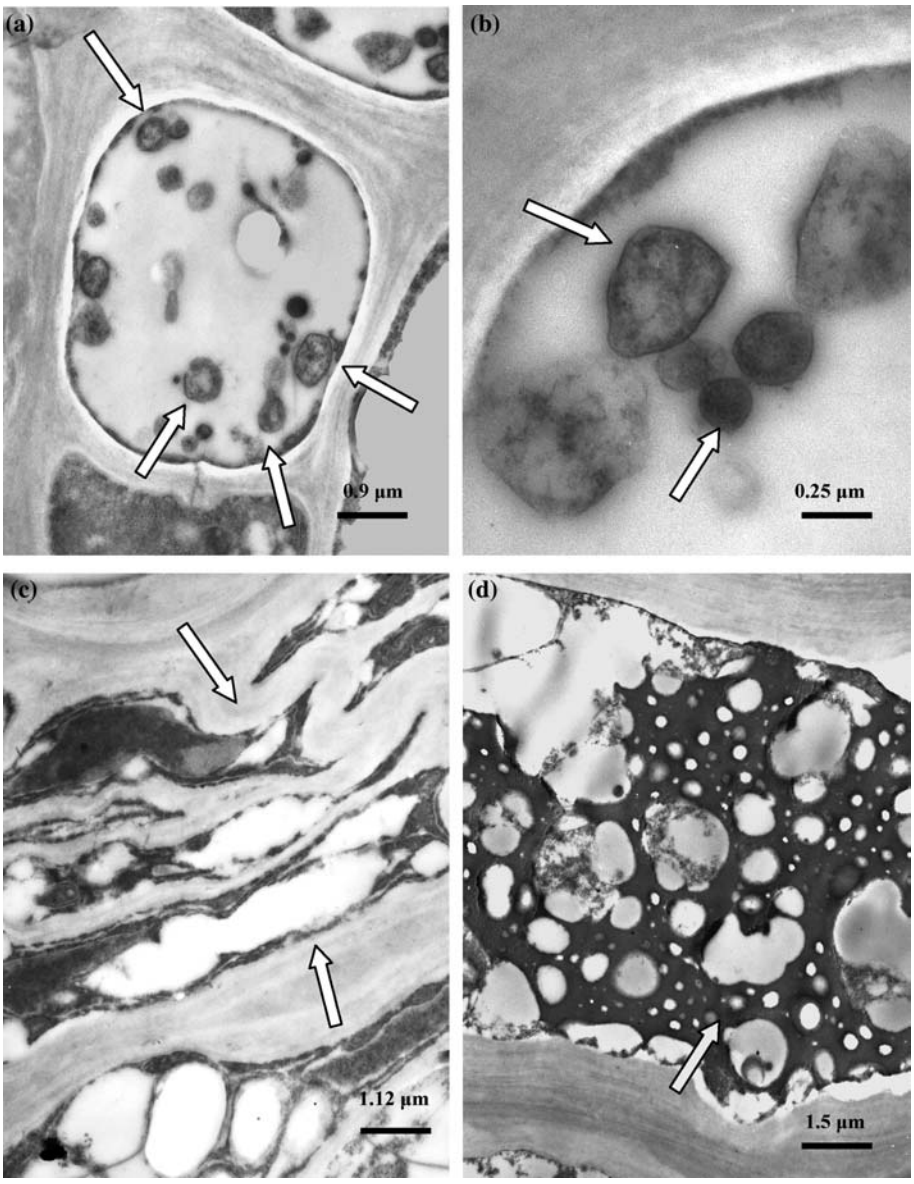

Figure 1. (a and b). Phytoplasmas ('*Ca.* Phytoplasma prunorum') (arrows) in the phloem of symptomatic leaf tissues. There are no deposits of electron-dense cerium perhydroxide. (c and d). The sieve tubes have often collapsed (c, arrows) and a number are necrotic or contain phenolic substances (d, arrows).

different from one another (data not shown), but there were significant differences in GPX activity (Figure 3a); with the following trend: symptomatic > recovered > asymptomatic. The GSH content followed the opposite trend: asymptomatic > recovered > symptomatic (Figure 3b), whereas, we did not observe differences in MDA concentrations between asymptomatic, symptomatic and recovered leaves (Figure 3c).

Discussion

Kison and Seemüller (2001) reported that different strains of '*Ca.* Phytoplasma prunorum' in apricot plants, can show huge differences in virulence and detection frequency associated with a lower phytoplasma concentration. It was thus chosen to use nested PCR to detect '*Ca.* Phytoplasma prunorum' in apricot plants, because this technique is

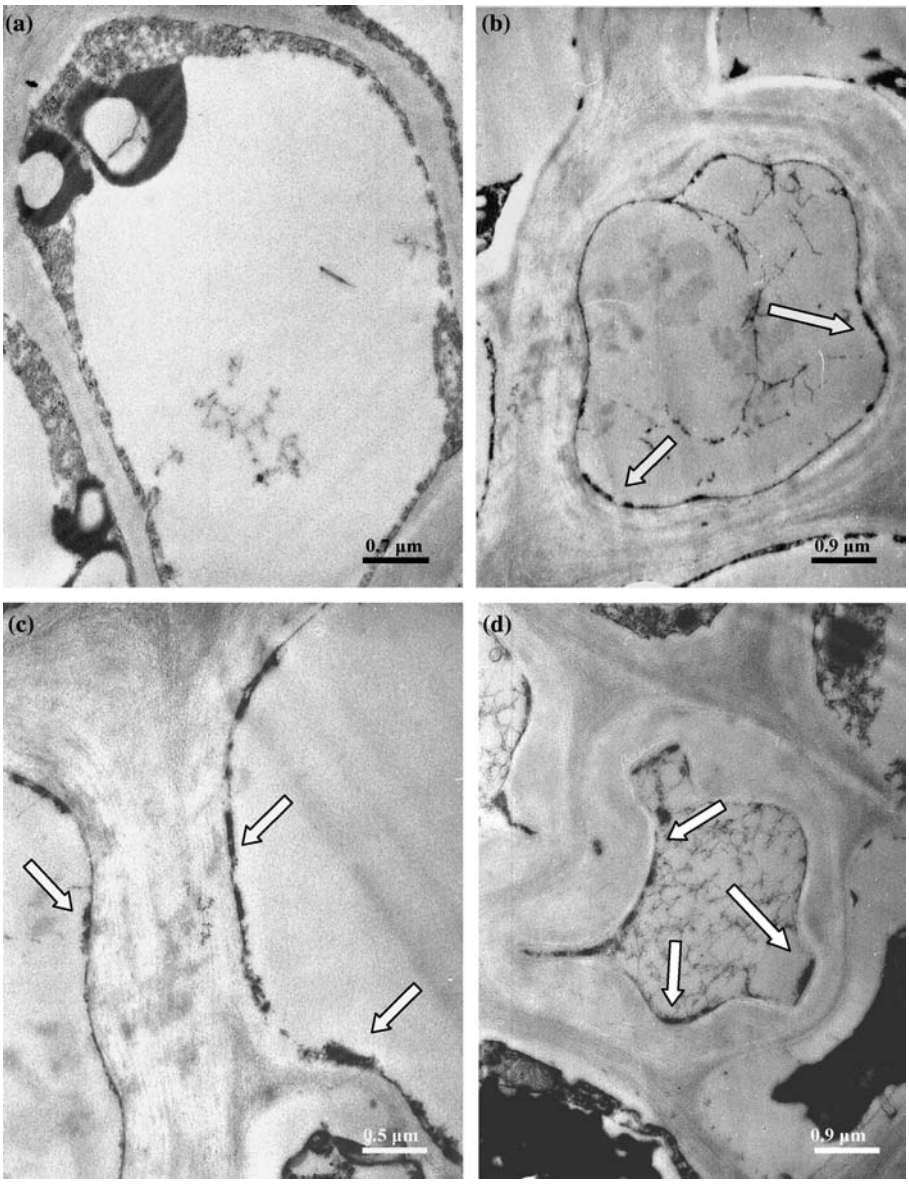

Figure 2. (a). Micrograph of asymptomatic leaf tissue of apricot: in the phloem, there are no precipitates of cerium perhydroxide, indicating the presence of H_2O_2. No phytoplasmas are revealed. (b–d) Micrographs of phloem cells of recovered apricot leaf tissue. No phytoplasmas are observed, cerium perhydroxide precipitates are localized in leaf tissue on the plasmalemma of sieve tubes (arrows).

highly sensitive and can detect phytoplasmas even in the case of scarcely concentrated/mild strains.

The nested PCR results reported indicate that all the analysed leaf samples from asymptomatic, symptomatic and recovered apricot trees were infected by '*Ca*. Phytoplasma prunorum'.

These results confirm the observations of Osler et al. (1999) on apricot plants, in which recovery appeared to be unrelated to the disappearance of the phytoplasma from the plant, as instead generally occurs in the canopy of apple trees and grapevines (Osler et al., 1993, 1999; Loi et al., 2002; Musetti et al., 2004).

The fact that asymptomatic apricot trees resulted positive to PCR analysis can be explained by hypothesizing the presence of '*Ca*. Phytoplasma

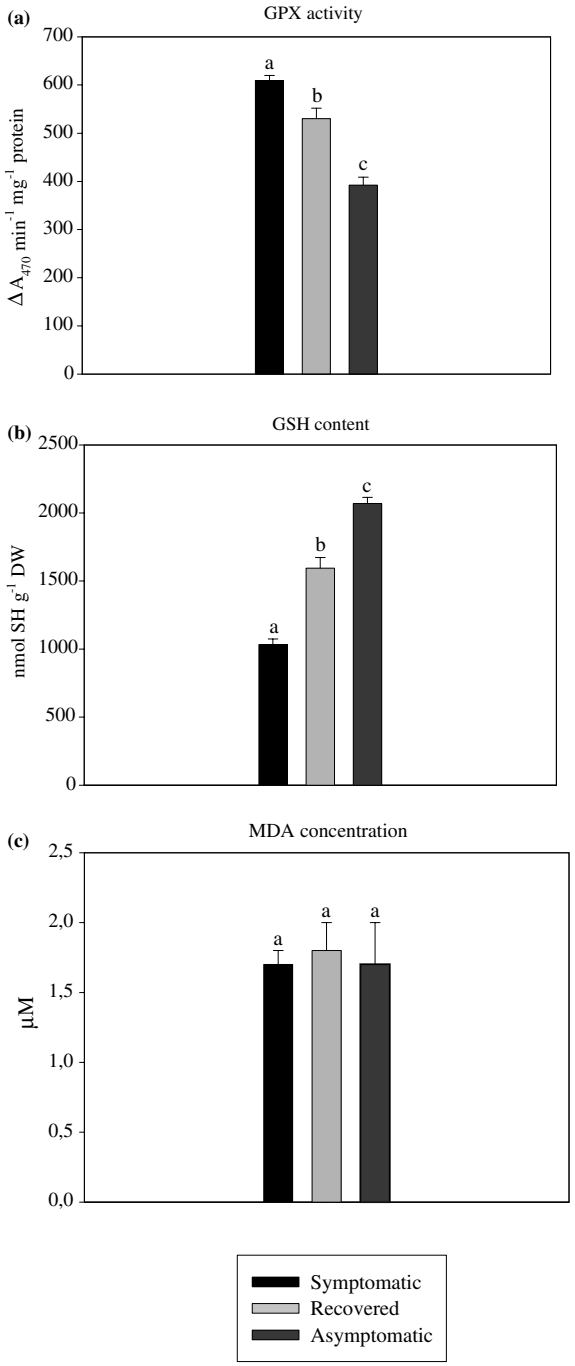

Figure 3. Guaiacol peroxidase (GPX) activity (a), reduced glutathione (GSH) content (b) and malondialdehyde (MDA) concentration (c) detected in ESFY-symptomatic, recovered and asymptomatic leaves of apricot trees. Different letters indicate significant differences at $P \leq 0.05$; $n = 3$; error bars denote SE.

prunorum' mild strains, combined with a lower phytoplasma concentration, as already reported by Kison and Seemüller (2001).

TEM observations of leaf tissues from symptomatic, asymptomatic and recovered apricot trees showed that H_2O_2 was present only in recovered tissues and was localized in the phloem. It is well known that H_2O_2 produced in response to pathogens has a direct antimicrobial effect and plays a role in several mechanisms involved in defence processes, including lignin production, lipid peroxidation, phytoalexin and pathogenesis related protein synthesis, hypersensitive response and induction of SAR (Lamb and Dixon, 1997; Wojtaszek, 1997; Bestwick et al., 1998; Vanacker et al., 2000).

Shetty et al. (2003) reported a significantly higher level of H_2O_2 in cultivars of wheat resistant to *Septoria tritici* than in susceptible ones. H_2O_2 also plays an important role in the oxidative burst, acting both as a local signal during the hypersensitive reaction (Martinez et al., 2000; Vanacker et al., 2000) and as a diffusible signal for the induction of cellular protective genes in adjacent healthy cells and tissues (Levine et al., 1994).

All these defence mechanisms perhaps contributed to the recovery phenomenon in the ESFY-infected apricot plants studied in this work.

In the experiments presented here, H_2O_2 overproduction does not occur in either asymptomatic leaves or symptomatic leaves, and this is possibly for contrasting reasons: asymptomatic leaves do not appear to manifest oxidative stress and thus have a lower ROS content than symptomatic leaves. In contrast, in symptomatic leaves, H_2O_2 production is low because it can be 'quenched' and counteracted by the induction of GPX activity and, at the same time, by the probable oxidation of GSH to GSSG. The GSH content is not depleted in asymptomatic leaves, presumably because of their low ROS content. Thus, only in the recovered material – possibly due to insufficient GPX induction and relatively low oxidation of GSH to GSSG, compared to symptomatic leaves – the ROS level appears to be sufficiently high (as shown by the abundant H_2O_2 in the tissues), to counteract the pathogen virulence.

Although GPX activity varied significantly between symptomatic, asymptomatic and recovered leaves, the localization of peroxidases by DAB did

not vary, confirming that peroxidases can be found in many compartments of plant cells (Asada, 1997; Bestwick et al., 1998; Andrews et al., 2002).

Furthermore, it could be speculated that the selective activation of GPX in response to infection by '*Ca*. Phytoplasma prunorum' might play a signaling role, aimed at triggering compensating/repair responses (Foyer et al., 1997). For instance, peroxidases can catalyze H_2O_2-dependent cross-linking of cell wall components and insolubilization of hydroxyproline-rich proteins (Iiyama et al., 1994; Wojtaszek, 1997). Such wall reinforcement can act as a mechanical barrier to pathogen penetration and restrict the diffusion of pathogen-synthesized toxins (Bestwick et al., 1998).

In conclusion, the prompt biosynthesis/activation of the above mentioned compounds could limit new infections generated by the same or other pathogens, thus enhancing tolerance to the diseases (Chamnongpol et al., 1998). In this sense, our results suggest that H_2O_2, related metabolites and enzymes appear to be involved in lessening both pathogen virulence and disease symptom expression, thereby playing a fundamental role in the recovery of ESFY-infected apricot plants.

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