Running title: Antioxidative enzymes during PPV infection

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Response of antioxidative enzymes to Plum Pox virus in two apricot cultivars

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Abstract

Recent evidence has indicated that activated oxygen species (AOS) may function as molecular signals in the induction of defence genes.

In this work the response of antioxidative enzymes to the plum pox potyvirus (PPV) was examined in two apricot (*Prunus armeniaca* L.) cultivars which behaved differently against PPV infection. In the inoculated resistant cultivar (Goldrich), a decrease in catalase (CAT) as well as an increase in total superoxide dismutase (SOD) and dehydroascorbate reductase (DHAR) activities were observed. Ascorbate peroxidase (APX), glutathione reductase (GR) and monodehydroascorbate reductase (MDHAR) did not change significantly in relation to non inoculated (control) plants. In the susceptible cultivar (Real Fino), inoculation with PPV brought about a decrease in catalase, SOD and GR, whereas a rise in APX, MDHAR and DHAR activities was found in comparison to non-inoculated (control) plants.

Apricot leaves contain only CuZn-SOD isozymes, which responded differently to PPV depending on the cultivar. Goldrich leaves contained 6 SODs and both SOD 1 and SOD 2 increased in the inoculated plants. In leaves from

Real Fino 5 SODs were detected and only SOD 5 was increased in inoculated plants.

The different behaviour of SODs (H_2O_2 -generating enzymes) and APX (an H_2O_2 -remover enzyme) in both cultivars, suggests an important role for H_2O_2 in the response to PPV of the resistant cultivar, in which no change in APX activity was observed. This result also points to further studies in order to determine if an alternative H_2O_2 -scavenging mechanism takes place in the resistant apricot cultivar exposed to PPV. On the other hand, the ability of the inoculated resistant cultivar to induce SOD 1 and SOD 2, as well as the important increase of DHAR seems to suggest a relationship between these activities and resistance to PPV.

This is the first report about the effect of PPV infection on the antioxidative enzymes of apricot plants. It opens the way for the further studies which are necessary for a better understanding of the role of antioxidative processes in viral infection by PPV in apricot plants.

Abbreviations: AOS, activated oxygen species; ASC, ascorbate, reduced form, ASC-GSH cycle, ascorbate-glutathione cycle; APX, ascorbate peroxidase; CAT, catalase; CuZn-SOD, copper, zinc-superoxide dismutase; DHA, ascorbate, oxidized form (dehydroascorbate); DHAR, dehydroascorbate reductase; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; PAGE, polyacrylamide

gel electrophoresis; $O_2^{\cdot-}$, superoxide radical; PPV, Plum pox virus; PCD, programmed cell death; SOD superoxide dismutase.

Introduction

Sharka, a disease caused by the plum pox virus (PPV) is the main virosis that affects apricot (*Prunus armeniaca* L.) species in the world, and one of the main factors restricting cultivation in the affected areas (Roy and Smith, 1994). In the short term, one way to control the spread of sharka in orchards is to rogue 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 apricot cultivars resistant to sharka is one of the main objectives of breeder. The evaluation of programme for PPV resistance, is time-consuming and very expensive (Martínez-Gómez and Dicenta 1999). Therefore, the search for 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.

The effects of various environmental stresses in plants are known to be mediated, at least in part, by an enhanced generation of activated oxygen species (AOS; $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$) (Hernández *et al.*, 1993, 1995; Alscher *et al.*, 1997; Creissen *et al.*, 1999). The antimicrobial response of both plants and animals is often accompanied by a coordinated activation of programmed cell death (PCD) and defence mechanism(s) (Greenberg, 1996).

One of the most peculiar events in the early phase of plant-pathogen interaction is the rapid and transient production of AOS ($O_2^{\cdot-}$, H_2O_2 and $\cdot OH$),

called the oxidative burst (Baker and Orlandi, 1995). It is thought that a plasma membrane-associated NAD(P)H is activated during the response of plants to pathogens (Jabs *et al.*, 1996). This results in the production of superoxide radicals ($O_2^{\cdot-}$), which dismutate spontaneously or via superoxide dismutase (SOD; EC 1.15.1.1) into H_2O_2 . Recent studies have indicated that AOS are key mediators of PCD during the hypersensitive response (HR) in *Arabidopsis*, bean and tobacco plants (Jabs *et al.*, 1996; Levine *et al.*, 1994; Draper, 1997). Characteristic features of the HR include the formation of a zone of dead cells around the infection site, the synthesis of salicylic acid and the accumulation of antimicrobial agents, such as pathogenesis-related (PR) proteins and phytoalexins (Bolwell *et al.*, 1995; Mehdy *et al.*, 1996; Hammound-Kosack and Jones, 1996). Hydrogen peroxide has been implicated in direct killing of pathogen cells and host cells and in the oxidative cross-linking of plant cell wall proteins to render the wall less digestible by microbial enzymes (Mehdy *et al.*, 1996). Moreover, AOS may function as part of a signal transduction pathway leading to the induction of PR protein and systemic resistance in infected and non-infected parts of the plant (Chen *et al.*, 1993; Green and Fluhr, 1995).

However, plants contain several mechanisms that detoxify $O_2^{\cdot-}$ and H_2O_2 and may inhibit PCD. To mitigate and repair damage initiated by AOS, plants have developed a complex antioxidant system. The primary components of this system include carotenoids, ascorbate, glutathione, and tocopherols and enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), 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; Foyer and Halliwell, 1976; Asada,

1992): 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.*, 1997).

The effect of fungal and TMV infections on the activity of antioxidative enzymes in plants has been previously reported (Doke and Ohashi, 1988; Vanacker *et al.*, 1998a,b; Fodor *et al.*, 1997). Changes in the isozyme pattern of peroxidase in *Nicotiana clevelandii* L. and *Chenopodium foetidum* L. plants associated with infection by PPV have been determined (Visedo *et al.*, 1990, 1991).

Isozyme profiles might be used as a reference when normal development of plants and pathological stages are compared, yielding valuable information about pathogenesis (Visedo *et al.*, 1990). No information about the effect of PPV infection on the activated oxygen-related enzymes in apricot plants is available. In order to determine the relationship between the activity of the antioxidative enzymes and the type of response to PPV infection in apricot we report here the changes in SOD isozymes, catalase and the ASC-GSH cycle enzymes due to the virus inoculation, in order to evaluate the usefulness of these antioxidant enzymes as biochemical markers of resistance of apricot plants to sharka virus.

Material and methods

Plant material

The apricot cultivars assayed were the North American cultivar Goldrich and the Spanish cultivar Real Fino. The GF305 peach, characterised by its high susceptibility to PPV, was used as rootstock. Plants were soil-grown in two litre pots, in controlled conditions in an insect-proof greenhouse, and fertilized with a complete nutrient solution (Martínez-Gómez and Dicenta, 1999).

Plum pox virus 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 representative of the Spanish PPV population (Asensio *et al.*, 1995), 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).

Evaluation of resistance to PPV

Young leaves from five different plants of each cultivar were assayed. The evaluation of resistance to PPV was carried out in controlled conditions as described by Martínez-Gómez and Dicenta (1999). The apricot cultivars Goldrich and Real Fino were grafted onto GF305 peach seedlings. The GF305 was previously inoculated by chip grafting with PPV and showed strong sharka symptoms on leaves. After grafting, the plants were first grown for 4 months in a sealed greenhouse with temperatures between 15°C and 30°C and relative humidity of around 70%. The plants were then exposed to a 2-month period in a cold chamber at 7°C in the dark, to induce artificial dormancy. Afterwards, they

were returned to the greenhouse for a second cycle of evaluation. The sharka symptoms in apricot leaves were evaluated on a scale from 0 (no symptoms) to 5 (maximum intensity). An ELISA-DASI (Cambra *et al.*, 1994) was also applied to leaves to corroborate the presence or absence of PPV.

Enzyme extraction and assays

In order to guarantee a high level of infection in the plants, leaf samples were taken during the second cycle. It has been shown that, in *Prunus* species, PPV usually needs at least two vegetative periods to produce symptoms (Martínez-Gómez and Dicenta, 2000).

Leaf samples were collected at two weeks following bud-breaking when symptoms on leaves of the susceptible cultivar Real Fino were strong.

All operations were performed at 0-4°C. Leaf material (2 g fresh weight), from both apricot cultivars, of non-infected control and inoculated plants, was homogenised with 4 ml of an ice-cold medium containing 50 mM K-phosphate (pH 7), 0.1 mM EDTA, 5 mM cysteine, 10% (w/v) PVP, 0.1 mM PMSF and 0.2% (v/v) Triton X-100. For the APX activity assay 20 mM Na-ascorbate were added to the extraction medium. The extracts were filtered through two layers of nylon cloth and centrifuged at 8000 g for 20 min at 4°C. The supernatant fractions were then filtered on Sephadex G-50 M PD10 columns (Pharmacia) equilibrated with the extraction medium.

APX, DHAR, MDHAR and GR activities were assayed as described in Jiménez *et al.* (1997). Enzyme activities were corrected for non-enzymatic rates and for interfering oxidation. For APX, the oxidation rate of ascorbate was estimated between 1.0 and 60 s after starting the reaction with the addition of

H₂O₂. Correction was made for the low non-enzymatic oxidation of ascorbate by H₂O₂. To determine MDHAR activity the monodehydroascorbate was generated by the ascorbate/ascorbate oxidase system. The rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) was subtracted from the initial monodehydroascorbate-dependent NADH oxidation rate (with ascorbate and ascorbate oxidase). For DHAR activity, the reaction rate was corrected for the non-enzymatic reduction of DHA by GSH. A 2% contribution to the absorbance by GSSG was also taken into account. Values due to GR activity were corrected for the small, non-enzymatic oxidation of NADPH by GSSG (Jiménez *et al.*, 1997).

Total SOD activity of the samples was determined according to McCord and Fridovich (1969). SOD isozymes were separated by PAGE on 10% gels using a Bio Rad Mini Protean II dual slab cell, and they were localised on the gels by the photochemical method of Weissiger and Fridovich (1973). Assignment of the different SOD isoforms (CuZn-SOD, Mn-SOD and Fe-SOD) was performed by selective inhibition with KCN or H₂O₂ (Hernández *et al.*, 1999). The isozyme activity was quantified by recording the transmittance of gels in a Shimadzu CS-9000 densitometer. Proteins were estimated according to Bradford (1976).

Results and discussion

Evaluation of Goldrich and Real Fino resistance to PPV

Table 1 shows the results obtained in the evaluation of the apricot cultivars in the two cycles of study. Young leaves of Goldrich cultivar did not show either symptoms or positive ELISA in any of the evaluated plants during the two cycles

of study and therefore, this cultivar was classified as resistant against the PPV isolate assayed. These results agree with those obtained by several authors who have regarded Goldrich as resistant to PPV (Dosba *et al.*, 1991, Karayiannis and Mainou, 1994). Conversely, the Spanish cultivar Real Fino appeared susceptible to the PPV isolate used. After the two cycles of study, all the evaluated plants showed leaf sharka symptoms and were ELISA+.

Response of antioxidative enzymes

Table 1 show that PPV inoculations led to the appearance of leaf symptoms in the susceptible apricot plants after two cycles of growth and pointed out the significant changes in antioxidative enzyme activity. Regarding to the SOD activity in the resistant and susceptible plants, a decrease of 40% of SOD activity with respect to the control occurred in susceptible plants but an increase, of 30%, took place in the resistant ones (Fig 1). The increase in SOD activity following pathogen attack may be required to catalyse the formation of H_2O_2 and prevent accumulaton of $O_2^{\cdot-}$ radicals. SOD activity might serve to reduce the risk of $\cdot OH$ radical formation by a Fenton-type reaction, which could be accompanied by enhanced superoxide production (Halliwell and Gutteridge, 1989). In barley plants, whole-leaf SOD activity did not change after fungal inoculation, but a significant increase in apoplastic SOD was found in both resistant and susceptible lines compared with non-inoculated controls (Vanacker *et al.*, 1998a).

Typically, after 10% PAGE of crude leaf extract, at least five distinct SOD isozymes, designated SOD 1-5, in order of increasing migration, could be detected in the sensitive apricot cultivar (Fig 2). In the resistant plants, six

different SOD isozymes were identified. The additional minor SOD had a lower electrophoretic mobility than SOD 3 of the sensitive plants and it was designated (for comparative purpose) as SOD 2'. In both cultivars, all the SOD bands were sensitive to both 2 mM KCN and 5 mM H₂O₂, indicating that they are Cu,Zn-containing superoxide dismutases (Fig 3).

The presence of only a Cu,Zn-SOD family has been reported also in leaves from *Quercus robur* L. plants, in which five Cu,Zn-SODs, localised in different cell compartments, including mitochondria and chloroplasts, were detected (Sehmer *et al.*, 1995).

The inoculation with PPV produced a differential response in the SOD isozyme pattern in both apricot cultivars. In the inoculated resistant cultivar, SOD 1 and SOD 2 showed increases of about 1.8 and 2.4-fold, respectively. However, the other isozymes did not show statistically significant changes (Table 2). Conversely, in the susceptible cultivar, only SOD 5 showed a significant increase (1.75-fold), whilst SODs 1, 2, 3 and 4 decreased (1.97-, 2.6-, 4.7- and 3.8-fold, respectively). In this cultivar no SOD 2' was found in control or inoculated plants (Table 2, Fig 2B). Nevertheless, we do not know the cellular localisation of the different SODs, and it could be very interesting to know in which cell compartment are localised those SOD isozymes that responded to PPV in each cultivar.

As for catalase, its specific enzyme activity decreased in relation to the controls in both cultivars, but the break-down was higher in the resistant (65%) than in the susceptible one (54%) (Fig 4). Regarding APX, in susceptible plants

infection by PPV increased leaf specific activity by 50%. However, no changes in APX activity were observed in the resistant cultivar, compared with non-inoculated controls. These results contrast to those shown by Vanacker *et al.* (1998a), using two barley cultivars differing in susceptibility to the fungus *Blumeria graminis*. In the resistant barley plants, the fungus inoculation caused a significant decrease in APX activity, whilst catalase did not show significant changes. However, in the susceptible barley cultivar, inoculation induced a massive increase in catalase activity (400%) and caused no significant changes in APX (Vanacker *et al.*, 1998a). These authors suggest that there is an inverse correlation between catalase induction in barley leaves and resistance to *B. Graminis* (Vanacker *et al.*, 1998a). However, an inverse correlation between APX induction and resistance to PPV seems to occur. These different responses could be due both to the plant specie used (herbaceous versus woody plants) and to the time-scales of the observed responses in the antioxidative enzymes. In this work the enzymatic analysis were made not at the first hours and/or days after inoculation but after two vegetative cycles of growth, because, this is the time required to assure an enough virus multiplication producing symptoms in the plants (Martinez-Gómez and Dicenta, 2000). It could be interesting to make other experiments at the first PPV stages in order to compare these data to those obtained in herbaceous plants.

It has been suggested recently that the inhibition of APX and catalase activities by salicylic acid during the response of plants to invading pathogens results in the accumulation of H₂O₂ and the acceleration of PCD (Draper, 1997). Recently, Mittler *et al.* (1998) reported that viral-induced PCD in tobacco is accompanied by the suppression of cytosolic APX expression, possibly at the

level of translation elongation. This suppression is likely to contribute to a reduction in the capability of cells to scavenge H_2O_2 , which in turn enables accumulation of this molecule. This finding supports the hypothesis that high H_2O_2 levels together with the suppression of antiperoxidative activity of the cell cause the activation of PCD and the defence mechanisms (Mittler *et al.*, 1998). In higher plants, catalase is localised mainly in peroxisomes (Huang *et al.*, 1983; del Río *et al.*, 1998). No measurements of H_2O_2 have been made in apricots plants. However, in both apricot cultivars studied here, catalase is inhibited by PPV inoculation. This decrease in catalase activity could contribute to a rise in peroxisomal H_2O_2 levels. Hydrogen peroxide could also diffuse through the peroxisomal membrane into the cytosol, in a manner similar to its previously observed peroxisomal and mitochondrial leakage into the cytosol during different stress conditions (Corpas *et al.*, 1993; Hernández *et al.*, 1993, Jiménez *et al.*, 1998a; del Río *et al.*, 1998). In this case, transient accumulation of H_2O_2 in apricot leaves after inoculation, due to the drop in catalase activity and/or probably to the oxidative burst (Draper, 1997; Mittler *et al.*, 1998), could increase the risk of oxidative injuries. But, H_2O_2 could also function as a cellular transduction signal in the plant hypersensitive disease resistance response (Levine *et al.*, 1994; Fink, 1998).

However, in the inoculated susceptible apricot cultivar, H_2O_2 may also be scavenged by the induced APX activity. This enzyme has a higher affinity for H_2O_2 than does catalase (Asada, 1992) and it has been shown to be present mainly in the cytosol (Gillham and Dodge, 1986) and chloroplasts (Foyer and Halliwell, 1976), although it is also present in the mitochondrial and peroxisomal

membranes (Jiménez *et al.*, 1997, 1998b; Mullen *et al.*, 1999). Although we do not know which APX isozyme was induced in the inoculated susceptible cultivar, this rise in total activity might have occurred too late in the response sequence to afford protection. Alternatively, APX induction might have caused a reduction in the level of H_2O_2 and thus it may have had a limited signal transduction effect, by effectively removing H_2O_2 as it was formed. These facts could facilitate further proliferation and spread of the virus in this cultivar. APX activity was not induced in the inoculated resistant cultivar, which together with the loss of catalase activity and the SOD induction, could allow an increased cellular H_2O_2 content that could induce defence genes (pathogenesis-related genes) in this resistant inoculated cultivar. These results could be related with the low virus concentration detected in leaves of the resistant cultivar, and they are compatibles with those obtained with the activated defence responses already reported in herbaceous plants (Chamnongpol *et al.*, 1996; Fodor *et al.*, 1997; Mittler *et al.*, 1998). Hydrogen peroxide-induced oxidation was found to cause expression of pathogenesis-related genes in transformed tobacco plants deficient in the major catalase isoform Cat 1 (Chamnongpol *et al.*, 1996).

In the inoculated susceptible cultivar, MDHAR increased up to 2-fold in relation to control plants. No inoculation-dependent increases in MDHAR activity were observed in the infected resistant cultivar, although its levels were similar to those in the inoculated sensitive cultivar. On the other hand, in non-inoculated plants, MDHAR activity was 2-fold higher in the resistant than in the sensitive cultivar (Fig 4).

Foliar DHAR activity was increased in inoculated plants of both apricot cultivars, but the rise was much higher in the resistant plants (300%) than in the sensitive ones (only 37%) (Fig 4). In whole extracts of barley no significant variations in DHAR activity were found, but an increase in apoplastic DHAR activity resulting from fungal inoculation was observed, this increase being higher in the resistant barley line (Vanacker *et al.*, 1998a). In the inoculated susceptible apricot cultivar, where APX showed a 50% rise, ascorbate could be oxidised via APX to a greater extent than in the inoculated resistant plants. Likewise, ascorbate reduction seems to have occurred mainly through the induced MDHAR activity, since in the susceptible cultivar DHAR increased only 37%, contrasting with the strong increase showed by DHAR in resistant plants. The strong increase found in DHAR activity in inoculated resistant apricot plants, in which APX activity did not change, may reflect the requirement for an efficient functioning of ascorbate reduction, which could be used as a substrate not only for APX but also for different enzymes, including cell wall peroxidases (Mehlhorn *et al.*, 1996; Takahama and Okini, 1992) and ascorbate oxidase (Esaka *et al.*, 1989). A strong plasmalemma-bound ascorbate-oxidising activity has been recently detected in *Phaseolus vulgaris* L. plasma membrane vesicles (Horemans *et al.*, 1996, 1997). Using ascorbate as an electron donor, cationic peroxidases might attack the hydrogen peroxide or free radicals. In this way, extracellular peroxidases could play a detoxifying role as oxidant scavengers in resistant apricot plants similar to that reported by Penel and Castillo (1989) for *Sedum album* L. upon exposure to ozone. Moreover, it has been suggested that the presence of ascorbate, together with apoplastic peroxidases, may regulate lignification processes in the cell wall (Takahama and Okini, 1992).

Stimulation of DHAR after inoculation in the resistant cultivar allows regeneration of ascorbate from dehydroascorbate, using the reducing ability of GSH. The recovery of GSH from its oxidised form is made possible by GR. As for this enzyme, its activity did not show statistically significant changes in the resistant inoculated apricot cultivar, but decreased in the inoculated susceptible plants (by 24%) (Fig 1). In tobacco Fodor *et al.* (1997) found a significant decrease in GR in the inoculated lower leaves 48 h after TMV inoculation. However, after 3 days GR activity had already increased, reaching 175% of the control value 7 days post-inoculation, which could contribute to a decline of the GSSG/total glutathione ratio in the infected leaves (Fodor *et al.*, 1997).

The strong increase in DHAR and the maintenance of GR activity in the resistant apricot cultivar suggest that in the infected leaves of these plants the regeneration of ascorbate could take place mainly via GSH. In this work we have not measured the ascorbate and glutathione contents, but the higher GR and DHAR activities in the inoculated resistant cultivar, as compared with the inoculated susceptible one, and the similar values found for MDHAR activity seem to indicate that the inoculated resistant plants have a higher capacity to regenerate ASC and GSH than do the inoculated susceptible plants.

The results are consistent with GR and DHAR acting against ASC oxidation in the inoculated resistant plants. However, the lack of an induction of GR activity concomitant with that of DHAR seems also to suggest that in inoculated resistant plants, the reduced glutathione necessary for DHAR activity must be supplied by another way, in addition to GR activity. This could include a higher rate of GSH synthesis and/or a more efficient transport of this antioxidant

to be used for the induced DHAR. The transport of ascorbate and glutathione through different cellular membranes has previously been described in plant systems (Schneider *et al.*, 1992; Foyer and Lelandais, 1996; Horemans *et al.*, 1997) and GSH, H_2O_2 and $\text{O}_2^{\cdot-}$, have been identified as messenger molecules in cellular signal transduction and also as factors in plant defence responses (Saran and Bors, 1989; Levine *et al.*, 1994, Foyer *et al.*, 1997, Karpinski *et al.*, 1997; Jiménez *et al.*, 1998a; Gómez *et al.*, 1999).

However, several redox proteins are able to perform reduction of DHA to ASC, including Kunitz-type proteinase inhibitor, protein disulphide isomerases, thioredoxins and thioredoxin reductase (Morell *et al.*, 1997; de Gara and Tommasi 1999). These are all able to utilise GSH, although these findings do not reject the possible involvement of other reducing metabolites in this *in vivo* reaction (de Pinto and de Gara, 1999).

Exogenously applied antioxidant often leads to increased stress resistance. An early report showed an antioxidative protective effect of ASC and GSH against necrotic viral infection (Farkas *et al.*, 1960). Vanacker *et al.* (1998a,b) showed an increase in apoplastic GSH in barley and oat lines resistant to the fungus *B. graminis*. However, for sensitive lines there was no increase, suggesting a possible relationship between GSH levels and resistance to the fungal infection. Similarly, in tobacco plants, Fodor *et al.* (1997) suggested an important role to GR, glutathione-S-transferase, SOD and the GSH level in the systemic acquired resistance. Several authors have observed a correlation between glutathione accumulation and pathogen resistance (Dron *et al.*, 1988; Vanacker *et al.*, 1998a,b; Gullner *et al.*, 1999). GSH, synthesised in leaf cells, is transported throughout the plant (Noctor and

Foyer, 1998) and therefore has been identified as a putative long-distance signalling molecule (Foyer *et al.*, 1997).

It is important to note that the results correspond to total activity values and do not reflect possible changes in isozyme populations, which could supply more detailed information on changes occurring in the different cell compartments. In fact, it has been suggested that changes in the levels of particular isoforms of antioxidant enzymes, rather than changes in their total activity may be more important in the stress response (Edwards *et al.*, 1994; Stevens *et al.*, 1997; Gómez *et al.*, 1999; Hernández *et al.*, 2000).

In this work, we show that PPV infection leads to important changes in the antioxidant systems in both apricot cultivars. The increase in SOD activity (SOD 1 and SOD 2) in the resistant cultivar may prevent accumulation of $O_2^{\cdot-}$ radicals and could reduce the risk of $\cdot OH$ radical formation. The induction of DHAR as well as the maintenance of GR and MDHAR contribute to an increased antioxidant capacity.

On the other hand, we also show a different pattern in the behaviour of APX and DHAR activities in both cultivars. DHAR, an ascorbate-regenerating enzyme, strongly increased in the inoculated resistant cultivar, but APX, an ascorbate-oxidising enzyme did not. In this way, we cannot discount the possibility that ascorbate could also be used by other enzymes different to APX (such as apoplastic class III peroxidases), which are also capable of oxidising ascorbate at the expenses of H_2O_2 (Mehlhorn *et al.*, 1996; Kvaratskhelia *et al.*, 1999).

In conclusion, the differential behaviour of SOD (an H_2O_2 -generating enzyme) and APX (an H_2O_2 -scavenging enzyme) suggests an important role for H_2O_2 in the response to PPV in the resistant cultivar. On the other hand, the ability of the inoculated resistant cultivar to induce the SOD 1 and SOD 2 isozymes and total SOD activity, as well as a higher DHAR induction, could be related to the resistance to PPV. These observations may be consistent with a role for SOD and ascorbate and glutathione metabolism in the response to PPV in apricot plants. More studies, using other apricot cultivars and a greater number of growth cycles, are needed, to check if the PPV infection elicits similar responses, in order to use these antioxidative enzymes (SOD 1, SOD 2 and DHAR) as possible markers of resistance to PPV.

Further studies using different subcellular fractions (apoplast, chloroplast, mitochondria and soluble fraction) from the two apricot cultivars could allow the discovery of which antioxidant isozymes are directly involved in the response to PPV. The current work opens the way for these studies.

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Table 1. Evaluation of resistance to PPV of 'Goldrich' and 'Real Fino' apricot cultivars. Number of plants with symptoms (and mean intensity) and number of plants ELISA + (and optical density at 405 nm in 60 min) during two cycles.

Cultivar	Evaluated plants	Cycle 1		Cycle 2	
		Symptoms ¹	ELISA ²	Symptoms ¹	ELISA ²
'Goldrich'	5	0 (0.0)	0 (0.28)	0 (0.0)	0 (0.24)
'Real Fino'	5	4 (2.3)	4 (1.55)	5 (2.3)	5 (1.68)

¹ Symptoms intensity on a scale from 0 (no symptoms) to 5 (maximum intensity).

² OD of healthy apricot control = 0.22.

Table 2. Effect of PPV inoculation on activity of leaf SOD isozymes (U mg⁻¹ protein) of susceptible (Real Fino) and resistant (Goldrich) apricot cultivars. Differences from control values were significant at: P < 0.05 (a); P < 0.01 (b); P < 0.001 (c) according the Duncan's Multiple Range Test; ND, not detected.

Cultivar	SOD 1	SOD 2	SOD 2'	SOD 3	SOD 4	SOD 5
Real Fino						
Control	12.78 ± 2.16	4.32 ± 0.73	ND	6.22 ± 1.05	10.66 ± 1.80	6.88 ± 1.16
Inoculated	6.48 ± 1.35 a	1.65 ± 0.34 a	ND	1.32 ± 0.23 b	2.76 ± 0.57 b	12.07 ± 2.51 a
Goldrich						
Control	8.40 ± 1.04	1.73 ± 0.21	1.32 ± 0.16	4.32 ± 0.53	6.29 ± 0.77	6.06 ± 0.74
Inoculated	15.20 ± 0.40 b	4.12 ± 0.01 c	1.19 ± 0.09	3.58 ± 0.03	6.40 ± 0.15	5.79 ± 0.14

Figure legends

Fig 1.- Response of SOD activity of apricot plants to PPV inoculation. Sc, control susceptible cultivar; Si, inoculated susceptible cultivar; Rc, control resistant cultivar; Ri, inoculated resistant cultivar.

Fig. 2.- A: SOD isozyme profiles in healthy and PPV infected leaves of apricot plants with different susceptibility to PPV. Control (A) and inoculated (B) susceptible cultivar. Control (C) and inoculated (D) resistant cultivar.
B: Densitometric analysis of non-denaturing activities gels of SOD isoforms from control and inoculated leaves of two apricot cultivars with different susceptibility to PPV. Control (A) and inoculated (B) susceptible cultivar. Control (C) and inoculated (D) resistant cultivar.

Fig. 3.- SOD isozyme identification after native PAGE on 10% acrylamide gels of apricot leaf extracts. Gels were stained for SOD activity and then scanned with a densitometer. 1, 3 and 5, cv. Real Fino. 2, 4 and 6, cv. Goldrich. Identification of SOD isoforms was performed by pre-incubation of gels with inhibitors. 1 and 2, controls (no inhibitors). 3 and 4, stained in the presence of 2 mM KCN. 5 and 6, stained in the presence of 5 mM H₂O₂.

Fig. 4.- Response of catalase activity and the ASC-GSH cycle enzymes of apricot plants to PPV inoculation. Sc, control susceptible cultivar; Si, inoculated susceptible cultivar; Rc, control resistant cultivar; Ri, inoculated resistant cultivar.

Fig 1

Fig 2

Fig 3

Fig 4