Sharka: How do plants respond to Plum pox virus infection?

Running title: Plant responses to sharka

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Highlights

PPV infection affect many different metabolic pathways including photosynthesis, photorespiration and antioxidant metabolism, leading to changes at physiological and biochemical levels as well as in protein and gene expression.

Abstract

Plum pox virus (PPV), the causal agent of Sharka disease, is one of the most studied plant viruses, and major advances in detection techniques, genome characterization and organization, gene expression, transmission and in the description of candidate genes involved in PPV resistance have been described. However, information concerning the plant response to PPV infection is very scarce. In this review we provide an updated summary of the research carried out to date in order to elucidate how plants cope with PPV-infection and their response at different levels, including the physiological, biochemical, proteomic and genetic levels. The knowledge about how plants respond to PPV infection can contribute to develop new strategies to cope with this disease. Due to the fact that PPV induce an oxidative stress in plants, the bio-fortification of the antioxidative defences, by classical or biotechnological approaches, would be a useful tool to cope with PPV infection. Nevertheless, there are still some gaps in knowledge related to PPV-plant interaction that remain to be filled, such as the effect of PPV on the hormonal profile of the plant or on the plant metabolome.

Keywords: antioxidant machinery, biochemical responses, oxidative stress, physiological responses, *Plum pox virus*, proteomic analysis, *Prunus*, sharka

Introduction

Sharka disease is considered one of the most detrimental diseases affecting many stone fruits and is also among the most studied viral diseases in the world (Scholthof *et al.,* 2011). The causal agent of sharka is *Plum pox virus* (PPV), belonging to the *Potyvirus* genus within the family *Potyviridae.* PPV isolates can be sub-divided into at least eight strains based on phylogenetic analyses, although new PPV isolates are continuously being collected worldwide (García *et al.,* 2014; James *et al.,* 2013; İlbağı *et al.,* 2014). Among these strains, PPV D and M are the most economically important and widespread (García *et al.,* 2014; James *et al.,* 2013).

During the last several decades, sharka disease has had a significant agronomic impact and has resulted in major economic losses, affecting mostly the Prunus genus (Cambra *et al.,* 2006). Indeed, since its first description in Bulgaria, in 1917(Atanasoff, 1932), sharka disease has spread from the European continent towards the most important Prunus-growing areas around the world, with the exception of Australia, New Zealand, South Africa and California (USA) (Garcia & Cambra, 2007). PPV has spread over long distances through the introduction of infected propagative plant material followed by local dispersion by aphids in a non-persistent manner. Strategies used to control the dispersion of the disease include the use of certificated PPV-free plant material, periodic surveys of orchards and the eradication of diseased trees. The reduction of the aphid vector in orchards by insecticide treatment is not effective against non-persistent viruses such as PPV. Disease control is very difficult, however, because sharka symptoms are highly dependent on both environmental conditions (temperature, age of the trees, etc.) and the sensitivity of the host plant. Typical sharka symptoms include chlorotic spots or rings, vein clearing and distortion on leaves, necrotic areas under shallow pale rings and deformation on fruits (Fig. 1) (Sochor *et al.,* 2012). In addition, fruits may drop prematurely, reducing both fruit quality and yield. In sharka-affected countries, the yield reduction of infected trees as well as the disease symptoms on fruits that make them unmarketable must be added to the costs of control, surveillance, and diagnostic and eradication programs. In 2006, it was estimated that sharka had cost a total of around 10.000 million euros over the previous 30 years worldwide (Cambra *et al.,* 2006, Barba *et al.,* 2011).

Most of the works on PPV have focused on its biological features, genome organization, vectors, host plants, distribution, and serological and molecular variability

(Sochor *et al.,* 2012; James *et al.,* 2013; Garcia *et al.,* 2014). The existing research concerning sharka disease includes the following three main topics: rapid and accurate diagnosis, the identification of sources of resistance and the obtention of new PPV-resistant varieties through classical or biotechnological breeding techniques. The diagnostic methods have included graft inoculation in GF305 peach (Martinez-Gómez and Dicenta, 2000); serological and immunological detection using PPV-specific antibodies (Vidal et al. 2012; Maejima *et al.,* 2014); and PCR-based methods (Olmos *et al.,* 2002). The GF305 peach cultivar, characterized by its great susceptibility to PPV, is usually used in PPV resistance tests on *Prunus*, both under *in vivo* (Martínez-Gómez and Dicenta, 2000) and *in vitro* conditions (Clemente-Moreno *et al.,* 2011; Monticelli *et al.,* 2012). It is important to note, however, that these testing methods have several limitations, particularly due to the irregular distribution of PPV in plant tissues. For more information about the diagnosis and detection of PPV, recent reviews such as those by Sochor *et al.* (2012) and García *et al.* (2014) are suggested.

To date, very few sources of PPV resistance have been identified in stone fruit species. Nevertheless, breeding programmes developed since the 1980s have characterized several PPV-resistant cultivars from different Prunus species, with most occurring in *P. armeniaca* (apricot) originating from North America (Martinez-Gomez and Dicenta, 2000), but also in cherry, almond (Rubio et al., 2005) and more recently in plum (Bozhkova and Milusheva, 2013). In peach, however, the most economically important Prunus specie, no source of resistance has been found in spite of the considerable effort carried out by breeding programs (Rubio et al., 2012). In the absence of resistant cultivars, tolerant cultivars, which display an active defence response resulting in localized cell death and symptomless fruit, have been used in some breeding programmes (Hartmann, 1998). The incorporation of resistance genes from other related wild species has also become a useful alternative. For example, the induction of resistance to PPV in "GF305" peach by "Garrigues" almond grafting has recently been reported (Rubio et al., 2013), suggesting that "Garrigues" grafting could be used as a natural vaccine against PPV in peach, although more studies are necessary to identify the resistance factors and understand how they work (Rubio et al., 2013).

The development of new methods, including the identification of molecular markers for resistance, would be very useful for breeding program. New molecular markers would help to select PPV-resistant sources. Rubio et al. (2014) described

simple sequence repeat (SSR) resistance markers linked to PPV resistance. These authors suggest that the use of homozygous resistant parents for SSR alleles with good agronomic characteristics, would improve the efficiency of breeding programs. However other authors suggest that marker-assisted breeding results could not be enough to select PPV- resistant sources (Decroocq et al., 2014). On the other hand, genetic engineering constitutes a faster reliable approach for inducing resistance to PPV. In recent years, PPV-resistant transgenic plants have been obtained using the expression of viral-derived sequences, including hairpin-containing viral transgenes (Di Nicola *et al.,* 2014; Di Nicola-Negri *et al.,* 2005; Ilardi and Di Nicola-Negri, 2011), and single-chain variable fragments specific to the NIb RNA PPV replicase (Gil et al., 2011). However, the regeneration and transformation of Prunus species is still difficult and is limited to a few genotypes (Petri and Burgos, 2005; Petri *et al.,* 2008).

Paradoxically, versus all the knowledge on PPV-based approaches, which have recently been discussed in different reviews (Sochor *et al.,* 2012; García *et al.,* 2014), few studies have focused on the PPV-infected plant responses. However, it has been described that PPV infection leads to metabolic changes at different levels. Knowledge concerning the different changes that occur at the physiological, biochemical, proteomic and genetic levels of infected plants versus resistant/susceptible plants may have a practical impact in terms of controlling sharka disease. In this review we provide an updated summary of the research carried out concerning this topic.

Physiological and Biochemical responses in PPV-infected plants

Knowledge concerning the physiological and biochemical responses of plants to PPV is very scarce. In early works, Visedo el al. (1990, 1991) studied changes in the isozyme pattern of peroxidase (POX) in *Nicotiana clevelandii* L. and *Chenopodium foetidum* L. plants in response to PPV infection. Ten years later, the first publication on the effect of PPV infection on the antioxidative metabolism of apricot leaves was published (Hernández et al. 2001). In this work, the response of the antioxidative enzymes to PPV-infection in two apricot cultivars, differing in their response to PPV infection, was studied in crude extracts. In the inoculated resistant cultivar (*Prunus armeniaca* cv. 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 (*P. armeniaca* cv. Real Fino), PPV infection produced a decrease in CAT, SOD and GR, whereas an increase in APX, MDHAR and DHAR activities was recorded in comparison to non-inoculated (control) plants. Based on the different behavior of SOD (H_2O_2-generating enzyme) and APX (H_2O_2-scavenging enzyme) in both cultivars, the authors suggested a role for H_2O_2 in the response to PPV in the resistant cultivar (Hernández *et al.,* 2001). In fact, a dual role for H_2O_2 has been widely described, for it is toxic in high concentrations, whereas in low concentrations it acts as a signalling molecule (second messenger) that mediates responses to various environmental stresses (Neill *et al.,* 2002). Moreover, reactive oxygen species (ROS) like H_2O_2 play a key role in signal transduction pathways in order to adjust cellular machinery to an altered condition (Jaspers and Kangasjärvi 2010; Miller *et al.,* 2010).

In later works, the effect of long-term PPV infection on the response of antioxidative metabolism at the subcellular level was studied in peach plants (*Prunus domestica* cv. GF305) and in apricot plants (Hernández *et al.,* 2004, 2006; Diaz-Vivancos *et al.,* 2006). These authors observed that infection by PPV produced an imbalance in the antioxidative metabolism and that PPV-susceptible species undergo oxidative stress in systemic leaves upon infection by the virus. In fact, an increase in oxidative stress parameters such as lipid peroxidation, protein oxidation and electrolyte leakage was observed in different PPV-susceptible peach plants (cv. GF305) and apricot plants (cv. Real Fino) (Fig. 2) (Hernandez *et al.,* 2004, 2006; Diaz-Vivancos *et al.,* 2006). Moreover, an accumulation of H_2O_2 was observed in leaves of these PPV-susceptible plants as well as a decrease in some antioxidant enzymes, mainly in chloroplasts (Fig. 3). In contrast, the response was somewhat different in the resistant apricot cultivar (*P. armeniaca* cv. SEO), since the PPV infection produced an increase in some antioxidant enzymes in the apoplastic space and in soluble fractions (Fig. 3), suggesting a tightly controlled production of H_2O_2, a response correlated with the PPV-resistance exhibited by this apricot cultivar (Hernandez *et al.,* 2006; Diaz-Vivancos *et al.,* 2006).

Similar studies to those described above have been performed in a susceptible pea cultivar (*Pisum sativum* L. cv. Alaska). In PPV-infected pea plants, an increase in oxidative stress parameters (Fig. 2) and in H_2O_2 content as well as an imbalance in the

antioxidative enzymatic system have also been described (Diaz-Vivancos *et al.,* 2008). These authors suggested ROS accumulation in PPV-infected plants, probably induced by a disturbance of the electron transport chain resulting from the alteration in the chloroplastic metabolism. In PPV-infected pea plants, therefore, chloroplasts could be a source of oxidative stress during viral disease development (Diaz-Vivancos *et al.,* 2008). In order to control H_2O_2 levels, an increase in the antioxidant capacity in the cytosol is very important because H_2O_2 can easily diffuse from cell organelles to the cytosol. The cytosolic antioxidant system thus seems to be very important in the response to oxidative stress induced by different abiotic and biotic disorders (Mittler and Zilinskas, 1994; Hernandez *et al.,* 2000; Faize *et al.,* 2011; Diaz-Vivancos *et al.,* 2013). The effect of virus infection on the antioxidative metabolism has been also studied in other plant-virus compatible or incompatible interactions, including other members from the *Potyviridae* family, such as *Potato Virus Y* (PVY). In PVY-tolerant as well as in PVY-resistant potato plants a significant increase in ionically-bound POX was observed in virus-inoculated leaves, suggesting that the fast response of these POXs may be central to a successful defense against viral pathogens (Milavec et al., 2008). In lower TMV-inoculated tobacco leaves, Fodor et al (1997) observed a decrease in some antioxidant enzymes, including APX, GR, GST and SOD, However, in the upper leaves an increase in GSH contents and GR, GST and SOD increased 10-14 days after TMV-inoculation of the lower leaves, concomitantly with the development of systemic acquired resistance (Fodor *et al.,* 1997). In susceptible *Dactylis glomerata* L. plants, the inoculation with *Cocksfoot motle virus* (CfMV) produced a biphasic response on the activity of antioxidants enzymes. At short-term (5 d) a decline in antioxidant enzymes was produced, following by its increase at long-term (up to 20 d) in response to the cellular damage (Li and Burrit, 2003). Song et al. (2009) studied the antioxidative response in CMV-infected cucumber and tomato leaves at subcellular level, and observed a general increase in the activity of SOD and the ASC-GSH cycle enzymes in chloroplasts, mitochondria and soluble fractions. More recently, molecular and biochemical markers including different POX isoenzymes, have been linked to the resistance to PVY and PVX in some potato cultivars (Mahfouze *et al.,* 2014).

PPV infection also produced alterations in the chlorophyll fluorescence parameters of susceptible plants. There are many studies that associate virus symptom expression with alterations in the chloroplast metabolism and function (Takahashi *et al.,*

1991; Rahoutei *et al.,* 2000; Pérez-Bueno et al. 2004; Hernández *et al.,* 2004; Diaz-Vivancos *et al.,* 2008). The mosaic or chlorotic symptoms in systemically virus-infected plants are attributable to chloroplast abnormalities. PPV infection produces an alteration in some chlorophyll fluorescence parameters, such as non-photochemical quenching (NPQ), photochemical quenching (qP) and the quantum yield of photosystem II (ϕPSII) in both susceptible peach (Fig. 4) and apricot cultivars (Hernández *et al.,* 2004, 2006). In symptomatic peach and pea leaves a slight increase in NPQ and its coefficient (qN) was accompanied by a decrease in qP and ϕPSII. However, an opposite response was observed in asymptomatic pea leaves (Fig. 4). In susceptible pea plants, the alteration in these parameters was correlated with a reduction in the amount of Rubisco and several polypeptides associated with PSII (Diaz-Vivancos *et al.,* 2008). A decrease in NPQ could reflect a diminished capacity for the safe dissipation of excess light energy, and therefore does not inhibit the production of harmful species, such as $^1O_2$, which would lead to worsened functioning and/or deterioration of the photosynthetic apparatus in the long term (Fryer *et al.,* 2002). Moreover, Perez-Bueno *et al.* (2006) linked the NPQ data with viral location by immunolocalization and changes in the ultrastructure of chloroplasts. These authors proposed the NPQ parameter as the best indicator of viral infection in the absence of symptoms. Similarly, the loss in qP (Fig. 4) is associated with an enhanced formation of $^1O_2$ (Foyer and Harbison, 1994). ROS accumulation along with a decline antioxidant enzymes level may thus be responsible for decreases in the photosynthetic efficiency of PPV-susceptible plants and the emergence and development of symptoms in these plants (Hernandez et al, 2006; Diaz-Vivancos et al, 2006).

Changes in cell ultrastructure induced in PPV-infected plants.

PPV infection caused an increased generation of ROS, imbalance in the antioxidative metabolism and proteome changes, mainly in the chloroplast (Hernandez *et al.,* 2004, 2006; Diaz-Vivancos *et al.,* 2008; Clemente-Moreno *et al.,* 2013). Moreover, the alteration of fluorescence parameters in PPV-infected plants is accompanied by modifications in the chloroplast ultrastructure. Almasi *et al.* (1996) described a slight deformation in the chloroplasts of PPV-infected *Nicotiana benthamiana* plants. Later, ultrastructure studies were carried out in peach, apricot and pea leaves. Cells from leaves showing sharka symptoms had fewer chloroplasts than

254 those observed in control plants, and these chloroplasts showed a disorganized structure
255 and dilated thylakoids, a reduced amount of grana and starch content, and an increase in
256 the number and size of plastoglobules (Fig. 5) (Hernandez *et al.,* 2006; Diaz-Vivancos
257 *et al.,* 2008; Clemente-Moreno *et al.,* 2013). The decrease in starch content in the
258 chloroplast can be explained by different hypotheses. PPV-infected leaves could have
259 increased demand for respiration, caused by activated defence responses and by the
260 requirements of the pathogen (Ayres et *al.*, 1996). In addition, PPV infection reduced
261 the expression of some proteins related with carbohydrate metabolism, such as aldolase
262 and ADP-glucose pyrophosphorylase (Clemente-Moreno *et al.,* 2013). Decreased
263 aldolase expression induced a decrease in starch (Haake *et al.,* 1999), whereas ADP-
264 glucose pyrophosphorylase is the major regulating enzyme in starch biosynthesis
265 (Tiessen *et al.*, 2002).

266 The presence of dilated thylakoids and an increase in plastoglobules (lipoprotein
267 particles inside chloroplasts) seem to be a general stress response and have been
268 previously described both under biotic and abiotic stress situations. An increase in the
269 number of plastoglobules due to an enhanced plastid lipid metabolism has been reported
270 in response to oxidative stress and during senescence (Austin II *et al.,* 2006). These
271 authors described that plastoglobules form linkage groups that are attached to each other
272 and remain continuous with the thylakoid membrane during oxidative stress and
273 senescence. The increase in the number and size of plastoglobules observed in PPV-
274 infected plants could thus be related to the establishment of oxidative stress during viral
275 disease development (Hernandez *et al.,* 2006; Diaz-Vivancos *et al.,* 2008; Clemente-
276 Moreno *et al.,* 2013). Similar effects have been reported in other plant-virus
277 interactions, such as in Zucchini yellow mosaic virus-infected pumpkin leaves, in which
278 the amount of plastoglobules increased significantly, whereas the amount of thylakoids
279 decreased (Zechmann *et al.*, 2003).

280

281 **Proteome changes induced in PPV-infected plants**

282 Changes in protein synthesis produced by virus infections is known long time
283 ago (Comacha and Sanger, 1982). Information about the effect of PPV infection on the
284 proteome of its hosts is very scarce. Diaz-Vivancos et al. (2006) published the first
285 information about the effect of PPV on differential apoplastic protein expression in
286 woody plants. The identification of proteins using MALDI-TOF (matrix-assisted laser

desorption/ionization-time of flight) and peptide mass fingerprinting analyses showed the induction of a thaumatin-like protein [a pathogenesis-related (PR) protein] as well as a decrease in mandelonitrile lyase [MDL, a flavoprotein involved in the catabolism of (R)-amygdaline] in peach apoplast due to PPV infection. However, most of the selected polypeptides showed no homology with known proteins (Diaz-Vivancos *et al.,* 2006). This fact emphasizes that, at least in Prunus, most of the functions of the apoplastic space remain unknown (Diaz-Vivancos *et al.,* 2006). These authors suggested that the increase in thaumatin could be mediated by H_2O_2, whose levels increased in the apoplast from infected peach leaves, and could be part of the response mechanism against unfavourable conditions in a state of weakness (Diaz-Vivancos *et al.,* 2006).

It has been described that infection by plant virus disturbs the PSII photochemistry and induces photoprotective mechanisms in order to preserve the integrity of this complex and to avoid photoinhibition in the host plant (Rahoutei et al. 2000; Pérez-Bueno *et al.,* 2004; Pineda *et al.,* 2008). The mosaic or chlorotic symptoms in plants with systemic infections are attributable to chloroplast abnormalities. As a result, there are many studies that associate virus symptom expression with the chloroplast function and metabolism (Rahoutei *et al.,* 2000; Pérez-Bueno *et al.,* 2004; Hernández *et al.,* 2004; Diaz-Vivancos *et al.,* 2008) (Table S1). Proteomic analyses carried out in pea leaves showed that most of the changes produced by PPV infection were mainly related to photosynthesis and carbohydrate metabolism. It seems that PPV infection has some effect on PSII, directly or indirectly, by decreasing the amount of Rubisco, the oxygen-evolving complex (OEC) and PSII stability factor proteins (Díaz-Vivancos *et al.,* 2008). In this work, the authors concluded that sharka symptoms observed in susceptible pea leaves could be due to an imbalance in antioxidant systems as well as to an increase in ROS generation in chloroplasts, probably induced by a disturbance in the electron transport chain, suggesting that chloroplasts can be a source of oxidative stress during viral disease development (Diaz-Vivancos *et al.,* 2008). In accordance with these observations, Perez-Bueno *et al.,* (2004) described that infection with PMMoV (Pepper Mild Mottle Virus) induced an inhibition of PSII electron transport, disturbing the oxygen-evolving complex (OEC). These authors observed a dramatic decrease in the contents of OEC polypeptides in isolated thylakoid membranes during the progression of the infection (Perez-Bueno *et al.,* 2004). Moreover, virus infection also affects the PSI. The potyvirus cylindrical inclusion (CI) protein interacts

with the PSI-K protein (Jimenez *et al.,* 2006). The co-expression of PPV CI has been shown to cause a decrease in the accumulation level of PSI-K and lead to a higher PPV accumulation, suggesting a role for CI-PSI-K interaction in PPV infection (Jimenez *et al.,* 2006).

More recently, differential protein expression was studied in PPV-infected peach plants (Clemente-Moreno *et al.,* 2013). This study showed that PPV infection reduced the abundance of proteins related to photosynthesis [ferredoxin-NADP(H) oxidoreductase; phosphoglycerate kinase; Rubisco (large subunit); ATPase (alpha subunit); and transketolase]; carbohydrate metabolism (fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, and ADP-glucose pyrophosphorylase); amino acid metabolism (aminomethyltransferase, aspartate transaminase, glutamine synthetase GSβ1, and serine hydroxymethyl transferase); and photorespiration (hydroxypyruvate reductase and catalase) (Clemente-Moreno *et al.,* 2013). Nevertheless, PPV infection increased the accumulation of other polypeptides associated with photosynthesis, such as glutamate-1-semialdehyde2 and 1-aminomutase, and also induced the expression of methionine synthase and some proteins associated with the response to stress, such as benzoquinone reductase and a putative chaperone clpb (Clemente-Moreno *et al.,* 2013). Regarding other potyvirus, it has been described that the infection of soybean plants with *soybean mosaic virus* (SMV) produced also variations in the abundance of different proteins including a down-regulation of proteins related with photosynthesis, carbohydrate metabolism or defence response (Rubisco, glyceraldehyde-3-phosphate dehydrogenase, PAL), and the induction of proteins related with ROS metabolism, respiration or energy transduction (Mn-SOD, lipoxygenase, NADPH isocitrate dehydrogenase, ATPase) (Yang et al., 2011).

PPV-induced changes in gene expression

Cloning genes that are up- or down-regulated by a viral infection can provide new insights into susceptibility to PPV or resistance mechanisms (Decroocq *et al.,* 2005). Different candidate genes have been described to be involved in the resistant or susceptible response to PPV in plants. The eukaryotic translation initiation factor eIF4E and genes involved in RNA silencing pathways are among the candidate genes of interest identified. The eIF4E factor and its isoforms [eIF(iso)4E] have been shown to

353 control susceptibility or resistance to virus infection in many plant species (Robaglia
354 and Caranta, 2006; Wang *et al.,* 2013). However, in most cases, inheritance of the
355 resistance associated with these factors has been shown to be recessive. On the other
356 hand, products of the RNA silencing pathway are known to be implied in antiviral
357 defence pathways (Vaucheret, 2008; Yu *et al.,* 2003).

358 Escalettes *et al.,* (2006) analyzed the effect of PPV infection on the gene
359 expression profile using cDNA-AFLP in two apricot cultivars, one partially resistant to
360 PPV (cv. Goldrich) and the other susceptible to the virus (cv. Screara). These authors
361 described that fragments coding for myosin, kinesin, transketolase and the ankyrin like-
362 protein were over-expressed in Goldrich and associated with susceptibility. Both
363 myosin and kinesin are involved in intracellular motile processes (Kinkema *et al.,* 1994)
364 and might play a role in the regulation of cell-to-cell transport. Transketolase takes part
365 in both the Calvin cycle and the oxidative pentose phosphate pathway (OPPP), playing
366 an important role in photosynthesis and in phenylpropanoid metabolism (Henkes *et al.,*
367 2001). These results agree with the data observed in a susceptible pea cultivar. In this
368 case, an increase in transketolase protein abundance was observed in PPV-infected
369 leaves at 15 days post-inoculation (Diaz-Vivancos *et al.,* 2008). The OPPP, besides its
370 important function in NADPH production, also supplies ribose-5-phosphate for the
371 synthesis of ribonucleotides, which can be required for virus replication in the cytosol
372 (Diaz-Vivancos *et al.,* 2008). Also, a cDNA coding for a putative class III chitinase was
373 repressed in infected plants from the resistant genotype and expressed in the susceptible
374 cultivar Screara, indicating that chitinases can be putatively involved in the compatible
375 interaction (Escalettes *et al.,* 2006). Plant chitinases are defence proteins induced by
376 environmental stress conditions (Collinge *et al.,* 1993). Moreover, PPV infection
377 increased the expression of NPR1 in peach GF305 under *in vitro* conditions (Clemente-
378 Moreno *et al.,* 2012). Furthermore, the salicylic acid (SA) related-induction or the
379 overexpression of NPR1 leads to an increase in the induction of PR gene expression and
380 enhanced disease resistance (Cao *et al.,* 1998; Kinkema *et al.,* 2000).

381 Microarray analysis of PPV-infected Arabidopsis plants showed the induction of
382 genes involved in soluble sugar, starch and amino acid metabolism; intracellular
383 membrane/membrane-bound organelles; chloroplasts; and protein fate. On the other
384 hand genes related to development/storage proteins, protein synthesis and translation,
385 and cell wall-associated components were down-regulated (Babu *et al.,* 2008). More

386 recently, Pagny *et al.* (2012) reported the identification of genomic regions from *A.*
387 *thaliana* associated with susceptibility to PPV, in particular to the long-distance
388 movement of the virus, such as RTM3-like TRAF domain-containing genes and MATH
389 and SHA3 genes.

390 The use of new approaches, such as the next-generation RNA sequencing (RNA-
391 seq), has been recently performed in order to achieve better understanding of the plant
392 responses to PPV. Rodamilans *et al.* (2014) compared PPV-infected and non-infected
393 samples by RNA-seq in order to identify genes involved in the resistance response.
394 According with this new approach, defence-related genes were generally up-regulated
395 whereas the expression of photosynthesis-related genes was repressed (Rodamilans *et*
396 *al.,* 2014). The down-regulation of genes associated with the photosynthetic pathways
397 agrees with the previously described data obtained by using proteomics and chlorophyll
398 fluorescence approaches. These authors suggest that some of the identified genes by
399 RNA-seq would be candidate genes for PPV resistance, establishing the basis for further
400 functional analyses (Rodamilans *et al.,* 2014).

401

402 **Biochemical based approaches to cope with PPV infection**

403 At present, most approaches involving transgenic process are very limited,
404 especially in the European Union, as they are not socially accepted even though their
405 potential for reducing pesticide input has been demonstrated (Lyon, Newton & Walters,
406 2007). Based on existing knowledge about the mechanisms of plant responses to
407 different environmental stresses, the use of compounds that could enhance plant defense
408 responses can be of interest (Beckers & Conrath, 2007). In fact, different compounds
409 have been used to prevent or reduce infection by different pathogens, including plant
410 viruses (Friedrich *et al.,* 1996; Lawton *et al.,* 1996; Gullner *et al.,* 1999; Anfoka, 2000;
411 Zechmann *et al.,* 2007; Clemente-Moreno *et al.,* 2010, 2012, 2013).

412 Some of these treatments used against PPV-infection include L-2-oxo-4-
413 thiazolidine-carboxylic acid (OTC) and (2,1,3)-benzothiadiazole (BTH). In 1981,
414 Williamson and Meister showed that the treatment of mice with OTC led to the
415 formation of cysteine, which increased the reduced glutathione (GSH) synthesis. Years
416 later, it was reported that OTC treatments also increased GSH levels and GSH-related
417 enzymes in several plant species, such as sorghum, spinach, tobacco, poplar, pumpkin,

418 pea and peach (Hilton *et al.,* 1990; Hausladen and Kunert 1990; Gullner *et al.,* 1999;
419 Komives *et al.,* 2003; Zechmann *et al.,* 2007; Clemente-Moreno *et al.,* 2010, 2012,
420 2013). These treatments were also reported to induce protection against different types
421 of viruses, including TMV, ZYMV and PPV (Gullner *et al.,* 1999; Zechmann *et al.,*
422 2007; Clemente-Moreno *et al.,* 2010). BTH is a functional analogue of salicylic acid
423 and has also been demonstrated to provide protection against different pathogens,
424 including fungus, bacteria and plant virus (Görlach *et al.,* 1996; Friedrich *et al.,* 1996;
425 Lawton *et al.,* 1996; Anfoka, 2000). However, studies on the use of OTC or BTH in
426 PPV-infected plants are limited to three papers published by our group based on
427 different models: herbaceous plants (pea) and woody plants (peach), both under *in vitro*
428 and greenhouse conditions (Clemente-Moreno *et al.,* 2010, 2012; 2013). In PPV-
429 infected plants, both OTC and BTH treatments reduced the severity of sharka, measured
430 as a percentage of leaves showing symptoms. In pea plants this response was correlated
431 with a higher redox state of GSH as well as with an increase in APX, POX and GSH-
432 related enzymes at the subcellular level (Clemente-Moreno *et al.,* 2010). In peach plants
433 OTC displayed better behavior against PPV than BTH. This response was associated
434 with the photosynthetic machinery and/or chloroplast metabolism protection in PPV-
435 infected peaches (Clemente-Moreno *et al.,* 2013). Changes in the proteomic profile of
436 PPV-infected peach plants did not take place in leaves from OTC-treated infected
437 plants. Moreover, OTC treatment induced some of the above-mentioned proteins whose
438 abundance was reduced by PPV infection (Clemente-Moreno *et al.,* 2013).

439 Furthermore, OTC stimulated plant growth in peach plants, measured as the
440 length of the main stem as well as by sprouting (number of leaves per plants)
441 (Clemente-Moreno *et al.,* 2013). In contrast, BTH had no significant effects on plant
442 growth, although BTH-treated peaches displayed a higher number of leaves than non-
443 treated plants (Clemente-Moreno *et al.,* 2013). Under *in vitro* conditions, low levels of
444 both BTH (5-10 μM) and OTC (10-50 μM) resulted in a significant increase in the
445 growth of peach and plum plantlets, and the effects were greater in PPV-infected peach
446 plantlets than in healthy peach plantlets (Clemente-Moreno *et al.,* 2012).

447

448 **Conclusions and perspectives**

449 The aim of this review has been to summarize plant responses to PPV infection,
450 given that PPV is one of the most devastating viral diseases among stone fruits. To date,

451 the scarce works attempting to elucidate these responses have shown that an imbalance
452 in the antioxidant machinery along with an accumulation of ROS might contribute to
453 the viral symptoms as well as to the deleterious effects of the PPV infection, being the
454 chloroplast and the photosynthetic machinery of the plant the most affected. However,
455 the response of plants to virus is somewhat different considering short- or long-term
456 responses (Li and Burrit, 2003; Diaz-Vivancos *et al.,* 2006, 2008; Hernández *et al.,*
457 2004, 2006), the plant-virus pathosystem and the environmental conditions. All these
458 factors make difficult understanding the plant responses to PPV, limiting the available
459 information about this topic. For example, the response of the antioxidants enzymes was
460 different in PPV-compatible peach and apricot interactions (Hernández et al., 2004,
461 2006). Although the increase in the antioxidants defences has been widely described in
462 incompatible plant-virus interactions (Fodor et al., 1997; Hernández et al., 2006;
463 Milavec et al., 2008), this response has been also reported in compatible plant-virus
464 interactions, such as peach-PPV, cucumber-CMV or tomato-CMV (Hernández et al.,
465 2004; Song et al 2009).

466 It is known that some natural or synthetic compounds can induce in plants a
467 more rapid and robust activation of defence responses to biotic or abiotic stress, and it is
468 often associated with development of stress tolerance (Conrath, 2011). Dissecting the
469 physiological and biochemical defence responses in genotypes which behaves
470 differently against PPV infection could lead to new approaches in breeding programs,
471 providing a better understanding of how plants cope with sharka disease.

472 Due to the fact that PPV infection induced an oxidative stress in susceptible
473 plants, biochemical or biotechnological approaches in order to reinforce the
474 antioxidative defences would constitute a useful tool to cope with PPV infection,
475 reducing PPV symptoms and economic loses. Nevertheless, several aspects of plant-
476 PPV interaction remain to be elucidated, such as the effect of PPV on the hormonal
477 profile of the plant or in the plant metabolome. Metabolomic analysis of susceptible and
478 resistant plants would lead to the identification of metabolites that might be specific
479 markers of PPV infection. This information could be used in the future not only to
480 identify infected plants in an early phase before the onset of symptoms but also to
481 identify new sources of resistance.

482

Supplementary Data

Table S1: Differential chloroplastic protein expression change in virus-plant interactions.

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Figure legends

Figure 1. Sharka symptoms in the leaves of PPV-infected (A) peach, (B) apricot, (C) plum and (D) pea.

Figure 2. Effect of PPV infection on oxidative stress parameters measured in leaves of susceptible and resistant plants. (A) Lipid peroxidation (measured as TBARS); (B) Protein oxidation (measured as carbonyl-proteins); (C) Electrolyte leakage (% from control values). GFc, peach GF305 control; GFi, inoculated GF305; RFc, apricot Real Fino control; RFi, inoculated Real Fino; Ac, pea Alaska control; Ai, inoculated Alaska; Sc, apricot SEO control; Si, inoculated SEO. [Adapted from Hernandez et al., 2004, 2006; Diaz-Vivancos et al., 2006, 2008].

Figure 3. Effect of PPV infection on the antioxidative metabolism at the subcellular level in leaves from susceptible and resistant apricot cultivars. PPV infection produced an oxidative stress at subcellular level in susceptible apricot (cv. Real Fino) that correlated with a general imbalance in the antioxidants defences in different cell compartments. However, in a resistant apricot cultivar (cv. SEO) no oxidative stress was observed that was accompanied by an increase in the antioxidative defences at subcellular level.

Figure 4. Effect of PPV infection on chlorophyll fluorescence parameters in leaves (C, control; A, asymptomatic; S, symptomatic) of peach (left images) and pea (right images). Images of the non-photochemical quenching (NPQ) and its coefficient (qN), the coefficient of photochemical quenching (qP) and the PSII quantum yield (ϕPSII) were obtained with a chlorophyll fluorometer (IMAGIM-PAM M-series, Heinz Walz, Germany). Infected peach leaves showed an increase in NPQ and qN and a decrease in qP and ϕPSII. This response was similar in symptomatic and asymptomatic leaves. Mature symptomatic infected pea leaves displayed a similar response to peach leaves. However, asymptomatic mature pea leaves showed the opposite response: reduced non-photochemical parameters and slightly increased photochemical quenching parameters.

Figure 5. Transmission electron microscopy of healthy (A and B) and PPV-infected (C and D) GF305 peach leaves. Chl, chloroplast; M, mitochondria; S, starch grain; V, vacuole.

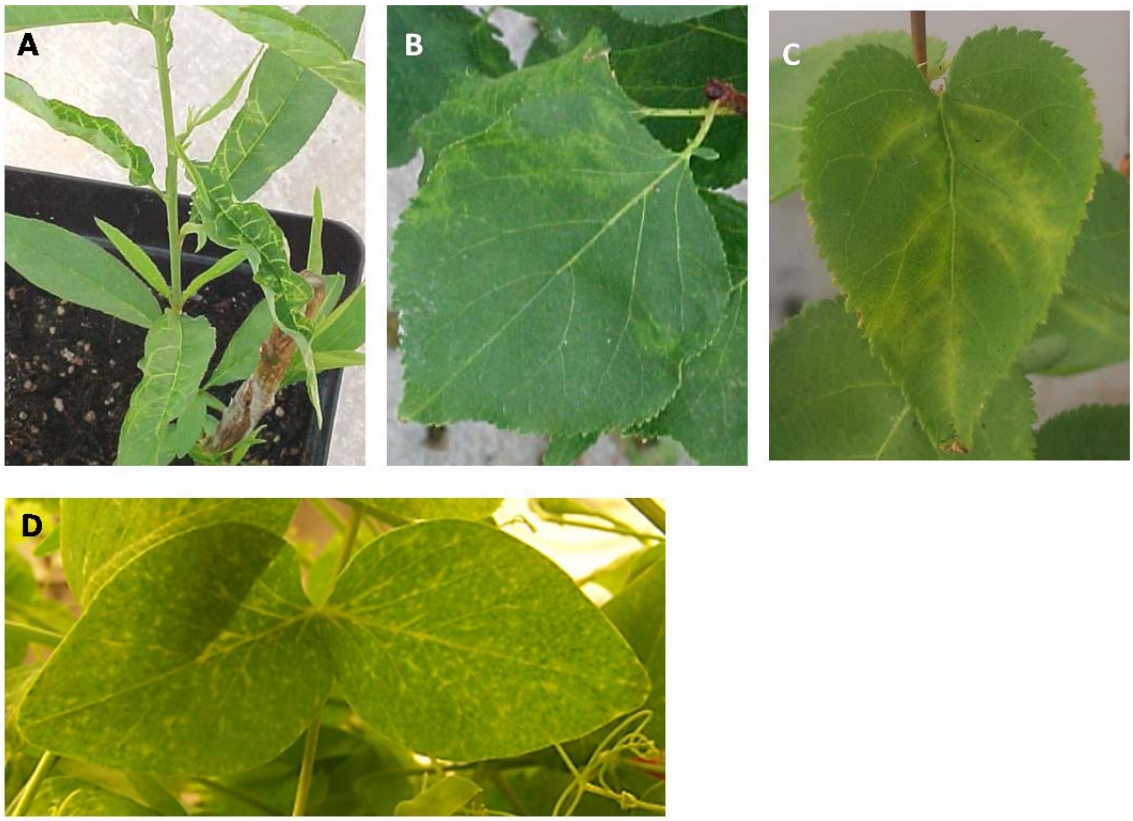

Fig1. Sharka symptoms in leaves of PPV infected (A) peach, (B) apricot, (C) plum and (D) pea.

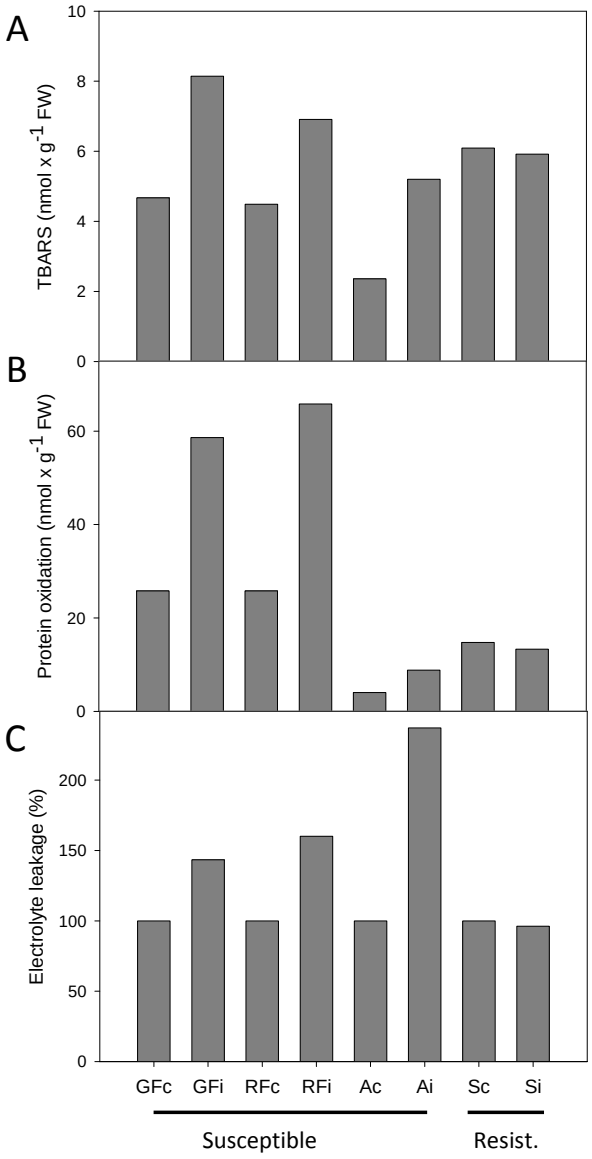

Fig 2. Effect of PPV infection on oxidative stress parameters measured in leaves of susceptible and resistant plants. (A) Lipid peroxidation (measured as TBARS); (B) Protein oxidation (measured as carbonyl-proteins); (C) Electrolyte leakage (% from control values). GFc, peach GF305 control; GFi, inoculated GF305; RFc, apricot Real Fino control; RFi, inoculated Real Fino; Ac, pea Alaska control; Ai, inoculated Alaska; Sc, apricot SEO control; Si, inoculated SEO. [Adapted from Hernandez et al., 2004, 2006; Diaz-Vivancos et al., 2006, 2008].

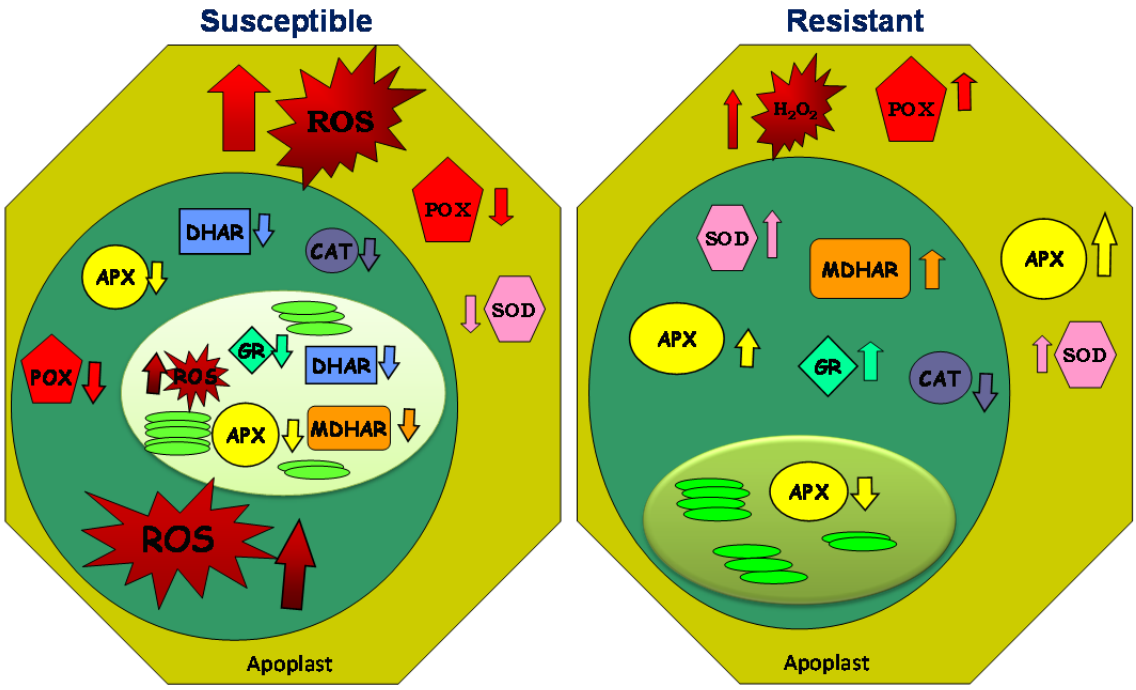

Fig 3. Effect of PPV infection on the antioxidative metabolism at subcellular level in leaves from susceptible and resistant apricot cultivars.

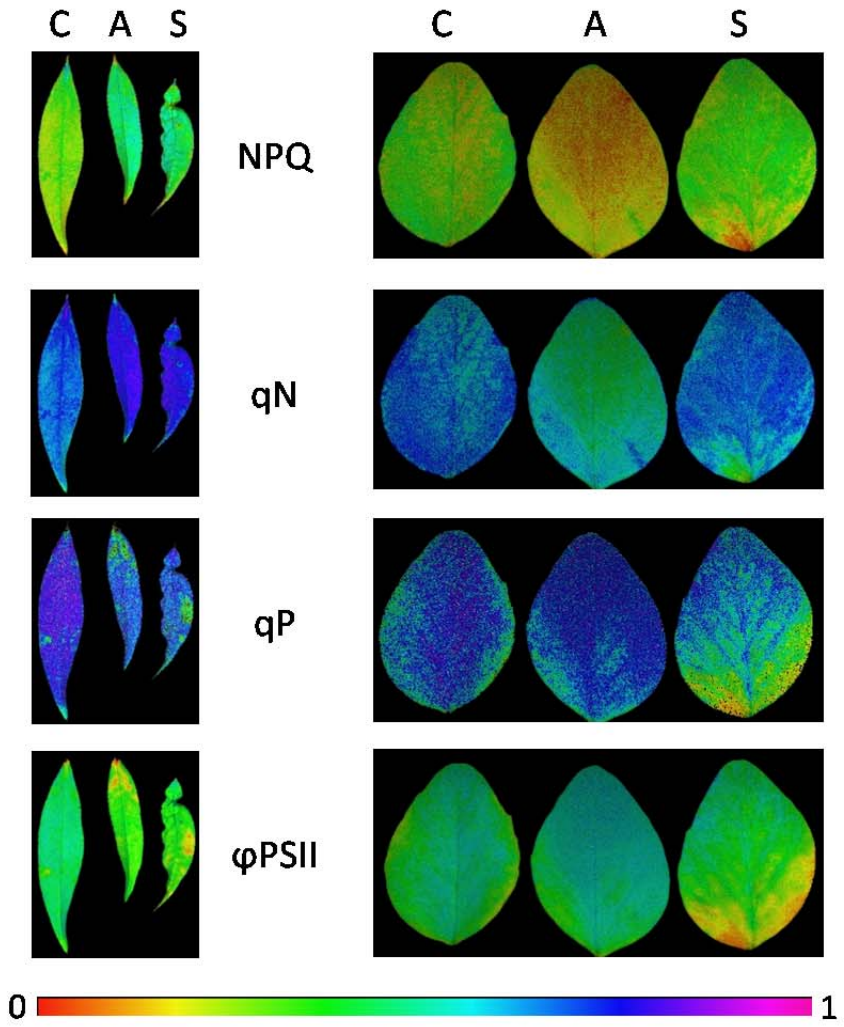

Fig. 4. Effect of PPV infection on chlorophyll fluorescence parameters in leaves (C, control; A, asymptomatic; S, symptomatic) of peach (left images) and pea (right images). Images of the non-photochemical quenching (NPQ) and its coefficient (qN), the coefficient of photochemical quenching (qP) and the PSII quantum yield (φPSII) were obtained with a chlorophyll fluorometer (IMAGIM-PAM M-series, Heinz Walz, Germany). Infected peach leaves showed an increase in NPQ and qN and a decrease in qP and φPSII, being this response similar in symptomatic and asymptomatic leaves. Mature symptomatic infected pea leaves displayed a similar response than peach leaves. However, asymptomatic mature pea leaves showed the opposite response: reduced non-photochemical parameters and slightly increased photochemical quenching parameters.

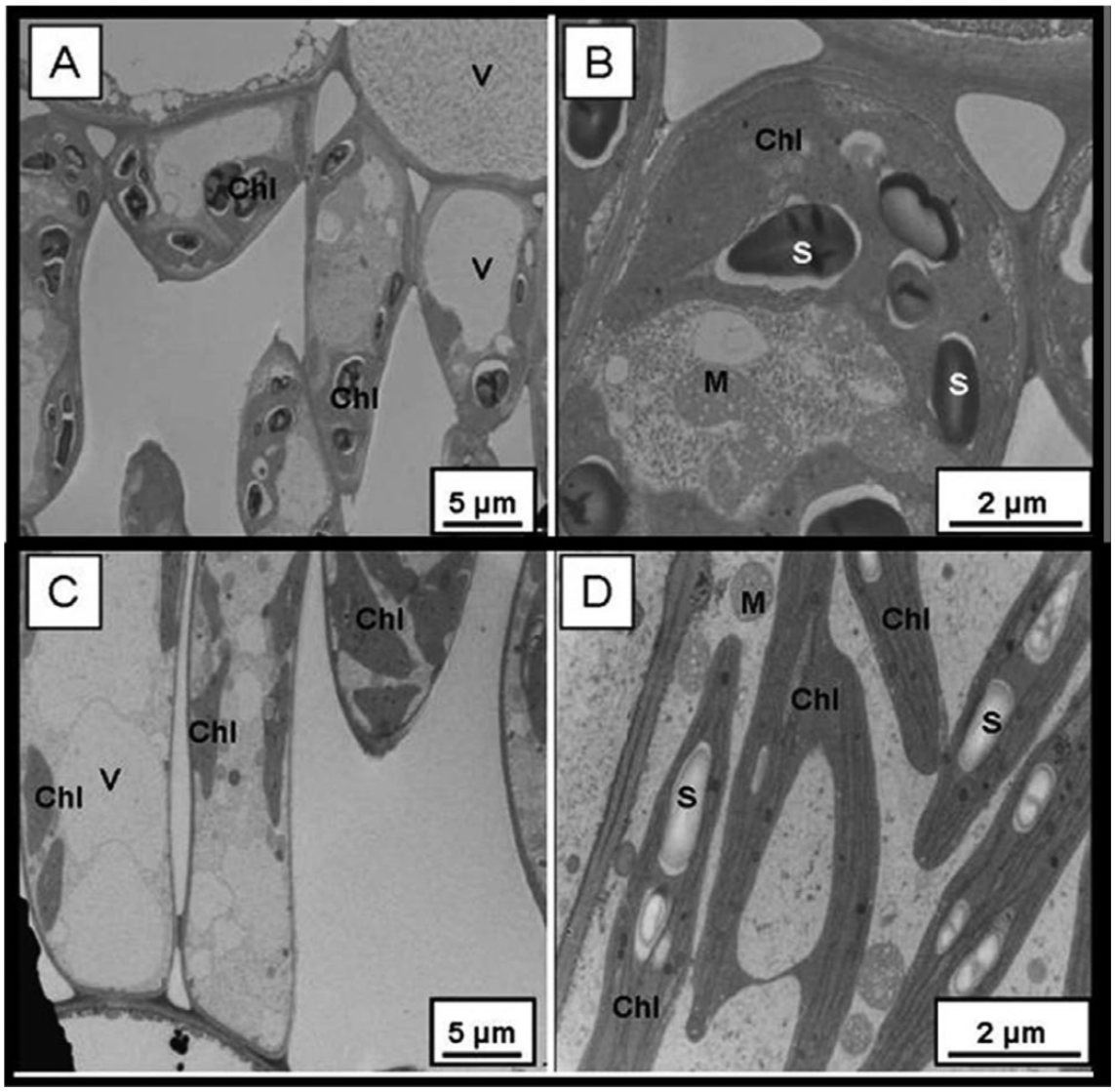

Fig. 5. Transmission electron microscopy of healthy (A and B) and PPV-infected (C and D) GF305 peach leaves. Chl, chloroplast; M, mitochondria; S, starch grain; V, vacuole. [From Clemente-Moreno et al., 2013. Chloroplast protection in plum pox virus-infected peach plants by l-2-oxo-4-thiazolidinecarboxylic acid treatments: effect in the proteome. Plant, Cell and Environment 36, 640–654. © 2012 Blackwell Publishing Ltd.]