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TESI DI DOTTORATO DI RICERCA

# Reactive oxygen species and oxylipin pathways: a unifying framework in oxidative stress.

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## Summary

Oxidative stress is involved in many aspects of plant physiology. In particular, reactive oxygen species (ROS) may represent harmful metabolites but, if strictly controlled, may also function as signal messengers able to induce defence responses. In this context, the role of antioxidant compounds is of outstanding importance, in order to strictly control ROS metabolism in the cell.

The aim of this thesis was to investigate two kinds of oxidative stress in different plant models: the first one concerns the involvement of ROS in apple trees affected by phytoplasmosis; the second one is focused on the oxidative stress in coffee beans during storage and germination.

Apple trees (*Malus domestica* Borkh) affected by apple proliferation (AP) caused by "*Candidatus* Phytoplasma Mali" can spontaneously recover from the disease. This phenomenon, known as recovery, consists in a spontaneous remission from disease symptoms in plants that were previously symptomatically affected by a given pathogen. After this process, phytoplasmas are no more detectable in the canopy, but are still present in the roots of apple trees.

In the first part of the present thesis it is shown that NAD(P)H peroxidases of leaf plasma membrane-enriched fractions exhibited a higer activity in samples from both AP-diseased and recovered plants. In addition, an increase in endogenous salicylic acid (SA) was characteristic of the symptomatic plants, since its content increased in leaf samples obtained from diseased apple trees. In agreement, phenylalanine ammonia lyase (PAL) activity, a key enzyme of the phenylpropanoid pathway, was increased too. Jasmonic acid (JA) increased only during recovery, in a phase subsequent to the pathological state, and in concomitance to a decline of SA. Oxylipin pathway, responsible for JA synthesis, was not induced during the development of AP-disease, but it appeared to be stimulated when the recovery occurred. Accordingly, lipoxygenase (LOX) activity, detected in plasma membrane-enriched fractions, showed an increase in apple leaves obtained from recovered plants. This enhancement was paralleled by an increase of hydroperoxide lyase (HPL) activity, detected in leaf microsomes, albeit the latter enzyme was activated in either the diseased or recovered conditions. Hence, a reciprocal antagonism between SA- and JA-pathways could be suggested as an effective mechanism by which apple plants react to phytoplasma invasions, thereby providing a suitable defence response leading to the establishment of the recovery phenomenon.

The objective of the second part of this thesis was the study of lipolytic activity of coffe seed (*Coffea arabica* L.), in either stored commercial coffee beans or germinating coffee seeds.

In four lots of commercial seeds from different provenances, phospholipase (PLase) A and lipase activities were assayed, and the caracterization of these lipolytic enzymes was performed. PLase exhibited two pH optima, at 6 and 7.5, suggesting the presence of two isoenzymes. Lipolytic activities were also evaluated in germinating green coffee from Colombia, either with or without parchment that represents the endocarp. The kinetics parameters ($K_M$ and $V_{max}$) of lipase activity were similar to what reported for lipases from Barbados nut (*Jatropha curcas* L.), whose seeds are classified as recalcitrant. The lipase activity was still present in seeds before imbibition and further induced during the germination process, which followed a biphasic behavior, similar in seeds with or without parchment, even though the phenomenon showed a delay in the former. Lipase activity gradually increased in coffee with parchment, in parallel with the delay in germination, indicating a general slower metabolism in such seeds. Western blot analysis, performed in the same samples, showed a similar expression pattern, indicating a biphasic trend for lipase activity. Gas chromatographic analysis of free fatty acid (FFA) content in the samples with and without parchment again confirmed the trend of lipolytic activity in the coffee seeds, since FFAs level increased more rapidly in samples without parchment, indicating a uncoordinated metabolism of lipid in the germinating seeds. Furthermore, the analysis of the antioxidant capacity indicates that the parchment was crucial for preventing the oxidation of the lipophilic fraction, being the parchment seeds less prone to oxidation. Therefore, it has been suggested that the parchment could act as a barrier to the oxygen fluxes towards the embryo/endosperm, thus limiting the production of ROS, allowing the germinating seeds to control the ROS level within the “oxidative window” for germination.

In conclusion, plants have evolved mechanisms able not only to control ROS, unavoidable by-products of living organisms, but also to fine tune their production in order to regulate many physiological processes. Indeed, in the present thesis it has been outlined that the strict control of oxidative stress could mediate the resistance to phytopathogen invasion in apple plants and the coordinated mobilization of lipid reserves during coffee seed germination.

# 1. Oxidative stress in plant cell

Redox homeostasis in plant cells is maintained by the appropriate balance between reactive oxygen species (ROS) generation and scavenging mechanisms (Mittler, 2002; Apel and Hirt, 2004). Under physiological conditions, ROS are continuously produced in plants as by-products of the metabolic activities in different compartments, mainly chloroplast, peroxisomes, mitochondria and cell wall. These oxygen derivatives possess a strong oxidizing potential that leads to damage for a variety of biological molecules and are, therefore, unwelcome by-products of normal metabolism in all aerobic organisms (Halliwell, 2006). During periods of biotic or abiotic stress, ROS levels can rise excessively, leading to an oxidative stress state (Apel and Hirt, 2004). To avoid dangerous damage, plants have developed an elaborate system to control cellular ROS concentrations (Mittler et al., 2011). In addition, plants have evolved a way to utilize lower concentrations of ROS, as signalling molecules, for a number of regulated processes during growth and development, like cell elongation (Foreman et al., 2003) and differentiation (Tsukagoshi et al., 2010), as well as in responses to a variety of environmental stimuli (Dat et al., 2000; Gapper and Dolan, 2006).

Therefore, it has been recognized that increases in ROS levels not only have toxic effects, inducing cell death by the oxidation of cellular components, but can also constitute a crucial signal to balance information between metabolism and the environment (Foyer and Noctor, 2005). The use of ROS, as signalling molecules, by plant cells suggests that, during the course of evolution, plants were able to achieve a high degree of control over ROS toxicity and have developed the ability to use ROS as signalling molecules (Mittler et al., 2004). The cells have the capacity to detoxify or scavenge ROS using a network of scavenging enzymes and antioxidant compounds found in almost all cellular compartments. This network enables cells to maintain a nontoxic steady-state level of ROS, while allowing the transient accumulation of ROS, in particular subcellular locations, acting as signals (Suzuki et al., 2011).

## *1.1 Reactive oxygen species (ROS)*

The term ROS defines all the products derived from reduction of molecular oxygen, either radicalic - atoms or molecules, electrically charged or neutral, with unpaired electrons in their structure - or not radicalic species, involved in the production of the radicalic form (Halliwell and Whiteman, 2004). Molecular oxygen is itself a free radical, since it has two unpaired electrons, with the same spin quantum number. This is the most stable state, or

ground state of oxygen, and is the form present in the air. Oxygen is a potent oxidizing agent but, to oxidize a nonradical atom or molecule, a triplet oxygen molecule would need to react with a partner that provides a pair of electrons with parallel spins that could fit into free electron orbitals. However, pairs of electrons typically have opposite spins, and this imposes a restriction on the reaction of triplet molecular oxygen with most organic molecules. Ground state oxygen may be converted to the much more reactive ROS forms either by energy transfer or by electron transfer reaction. The former leads to the formation of singlet oxygen, whereas the latter results in the sequential reduction to superoxide, hydrogen peroxide, and hydroxyl radical (Apel and Hirt, 2004). The most reactive form of $O_2$, singlet oxygens ($^1O_2$), can be generated by an input of energy that rearranges the electrons (Fig. 1). In both forms of singlet oxygen the spin restriction is removed and the oxidizing ability greatly increased, allowing singlet oxygen to directly oxidize proteins, DNA, and lipids.

If a single electron is supplied to $O_2$, superoxide radical ($O_2^{\bullet -}$) is produced, which, with only one unpaired electron, is less reactive than $O_2$. Superoxide is present at low concentration in the cells of aerobic organisms, and could react with many biomolecules (proteins, lipids, fatty acids, polysaccharides) (Halliwell, 2006).

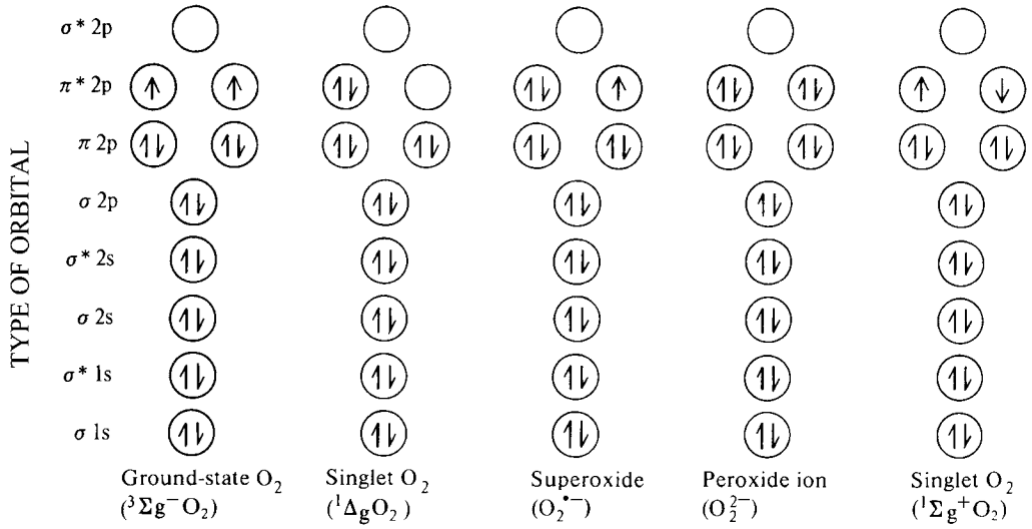


**Fig.1** Simplified version of bonding in the diatomic oxygen molecule and its derivative (*from Halliwell, 2006*).

Hydrogen peroxide ($H_2O_2$) is the intermediate molecule produced by a "two-electrons reduction" of oxygen. However, in biological systems, $H_2O_2$ may also be generated through a dismutation reaction, spontaneous or catalyzed by the superoxide dismutase (SOD):

$$2\ O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$H_2O_2$ is a non-radical form of oxygen, which is permeable to lipoproteic membranes, with a high toxicity against many cellular components, like sulfhydrilic residues of proteins. Hydrogen peroxide can be transported through specific aquaporins, homologues of the TIP (tonoplast intrinsic protein) and PIP (plasma membrane intrinsic protein) families (Bienert et al., 2007). The biological effect of $H_2O_2$ is mostly dependent on its concentration, but also on the site of production, the developmental stage of the plant, and previous exposures to different kinds of stress. Generally, $H_2O_2$ acts at low concentrations as a signalling molecule, while at higher concentrations it is responsible for the onset of cell death (Gechev and Hille, 2005). The further reduction of hydrogen peroxide ("three electron reduction state") produces the harmful hydroxyl radical ($HO^{\bullet}$). In cells, this ROS is also formed by the interaction of $H_2O_2$ with metal ions like $Fe^{2+}$/ $Fe^{3+}$ and $Cu^{+}$/$Cu^{2+}$, in the Fenton (or Haber-Weiss) reactions (Halliwell and Gutteridge, 1990):

$$O_2^{\bullet -} + Fe^{3+}\ (\text{or } Cu^{2+}) \rightarrow O_2 + Fe^{2+}\ (\text{or } Cu^{+})$$

$$H_2O_2 + Fe^{2+}\ (\text{or } Cu^{+}) \rightarrow HO^{\bullet} + OH^- + Fe^{3+}\ (\text{or } Cu^{2+})$$

$HO^{\bullet}$ is the most reactive of all ROS, because it can potentially react with all biological molecules, causing structural and functional damages, and cells have no enzymatic mechanisms to eliminate this product.

## 2. Cellular localization of ROS production

In photosynthetic tissues, the chloroplast is the major source of ROS, having the capacity to produce high amounts of $O_2^{\bullet -}$ and $H_2O_2$, especially during conditions of reduced rate of photosynthetic carbon fixation - a typical situation occurring in abiotic stress. In addition, chloroplasts can produce $^1O_2$ through excited chlorophyll molecules.

In plant mitochondria, ROS formation, mainly as $O_2^{\bullet-}$, is favoured when the electron transport chain becomes overreduced. The redox status of the mitochondrial electron transport chain (ETC) is an important indicator of the cell energy status and ROS, especially $O_2^{\bullet-}$ (from complexes I and III) and reduced ubiquinone pool are integral parts of this monitoring system.

Peroxisomes are probably the major site of intracellular ROS production. The main metabolic processes responsible for the generation of $H_2O_2$ in different type of peroxisomes are the photorespiratory glycolate oxidase reaction, the fatty acid β-oxidation, the enzymatic reaction of flavin oxidase, and the disproportionation of $O_2^{\bullet-}$. Increased production of $H_2O_2$ and $O_2^{\bullet-}$ in the peroxisomes leads to oxidative damage and possibly cell death, but small levels of $H_2O_2$ and $O_2^{\bullet-}$ could act as signal molecules, which mediate pathogen-induced programmed cell death (PCD) in plants. Therefore, it has been suggested that peroxisomes should be considered as cellular compartments with the capacity to generate and release important signal molecules such as $H_2O_2$, $O_2^{\bullet-}$ and $NO^{\bullet}$, which can contribute to the formation of a more integrated communication system among cell compartments (Gill and Tuteja, 2010).

## *2.1 Apoplastic ROS production*

NAD(P)H oxidase, known as the respiratory burst oxidase (RBO), catalyzes the production of $O_2^{\bullet-}$ by the one-electron reduction of molecular oxygen using NAD(P)H as an electron donor (Choi et al., 2007). RBO was initially described in mammalian neutrophils as a multicomponent complex mediating microbial killing (Torres et al., 2006). The enzymatic subunit of this oxidase ($gp91^{phox}$) transfers electrons to molecular oxygen to generate superoxide.

Plant homologs of NAD(P)H oxidases are called RBOH (Respiratoy burst oxidase homologs). Several reports demonstrate that members of the RBOH family mediate the production of apoplastic ROS during the defence responses, as well as in response to abiotic environmental and developmental cues (Torres and Dangl, 2005).

The plant RBOH constitute a multigenic family with ten genes in the model plant Arabidopsis (*Arabidopsis thaliana*) (AtRboh) (Mittler et al., 2004). All RBOH localize to the plasma membrane and present the same domain structure, with a core C-terminal region that contains the transmembrane domains and the functional oxidase domain responsible for superoxide generation (Fig. 2). However, plant RBOH have an additional N-terminal region, absent in the phagocyte oxidase $gp91^{phox}$, but present in other animal NAD(P)H oxidases,

such as NOX5 and Duox (Torres and Dangl, 2005). This N-terminal extension contains regulatory regions, such as calcium-binding EF-hands and phosphorylation domains, important for the function of the plant oxidases. RBOH also contain cytosolic FAD- and NADPH-binding domains, as well as six transmembrane helices with two hemes. The latter are required for electron transport, through membrane, to the extracellular acceptor $O_2$ and are linked to the third and the fifth transmembrane helices via histidine residues (Glyan'ko and Ischenko, 2010).

In recent years, several genetic studies have revealed that plant RBOH play a multitude of different signalling functions. RBOH-dependent ROS production has been associated with the establishment of plant defences in response to pathogens, often in association with the hypersensitive response (Torres et al., 2002).

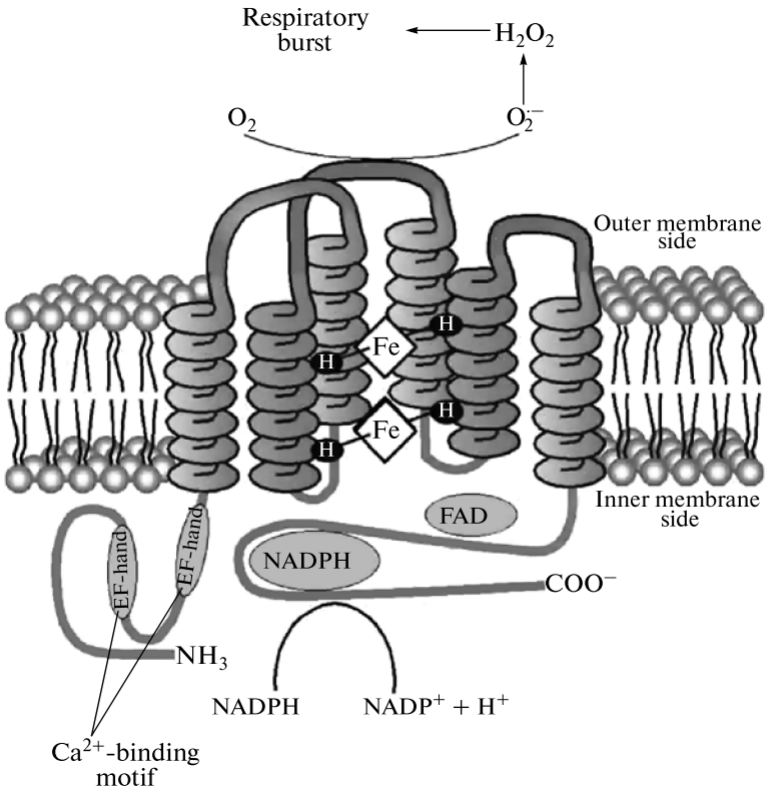


**Fig. 2** Extended scheme of the structure of plant NADPH oxidase (RBOH) (*from Glyan'ko and Ischenko, 2010*).

ROS have also been implicated in the regulation of cellular responses to stresses other than pathogen infection. For example, $H_2O_2$ acts as a second messenger for the induction of defence genes in response to systemin and jasmonate (JA) during wound responses (Orozco-Cardenas et al., 2001). Anti-sense transgenic mutations showed that ROS, produced by an RBOH, are required for the expression of certain wound-responsive genes (Sagi et al., 2004).

These findings suggest that ROS, produced by RBOH could influence metabolic balance, acting in several hormone signalling pathways (Orozco-Cardenas et al., 2001). Moreover, RBOH regulate signalling in response to abiotic stresses such as heat, drought, cold, high-light intensity, salinity or wounding (Kwak et al., 2003; Miller et al., 2009).

Progress in understanding the regulation of RBOH has focused in recent years on how the N-terminal region of RBOH plays a crucial role in the regulation of oxidase activity (Oda et al., 2010). A tight relationship between RBOH-dependent ROS production and calcium homeostasis has been revealed. A positive feedback amplification of these signals has been recognized, with calcium binding to the RBOH EF-hands and promoting ROS production, which subsequently activate calcium channels (Takeda et al., 2008; Ogasawara et al., 2008). Self-amplification loops, involving RBOH and phosphorylation, also contribute to the amplification of signals in defence to pathogens, wound responses and stomatal closure (Zhang et al., 2007; Asai et al., 2008; Jammes et al., 2009). Phosphorylation of residues, located at the N-terminal region of RBOH, by calcium-dependent protein kinases implies a crosstalk between calcium and phosphorylation in the regulation of ROS production (Kobayashi et al., 2007).

In addition to plant NAD(P)H oxidases, class III peroxidases (Prxs) have been proposed as alternative apoplastic producers of ROS. Prxs are heme-containing enzymes belonging to the superfamily of Prxs from plants, fungi, unicellular eukaryotes, and bacteria (Welinder, 1992; Passardi et al., 2007). These enzymes catalyze the reduction of $H_2O_2$ by taking electrons from various donor molecules such as phenolic compounds, lignin precursors, auxin, or secondary metabolites (Hiraga et al., 2001). Prxs are involved in a broad range of physiological processes throughout the plant life cycle, probably due to the high number of enzymatic isoforms (isoenzymes) and to the versatility of their enzyme-catalyzed reactions (Passardi et al., 2005). In higher plants, the number of isoenzymes is extremely high, corresponding to 73 genes of class III Prxs within the Arabidopsis genome. Several other isoforms can be generated by post-transcriptional and post-translational modifications (Welinder et al., 2002).

Plant Prxs are involved in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis, and metabolism of ROS and reactive nitrogen species. Like NAD(P)H oxidases, Prxs do not directly generate $H_2O_2$, but catalyze the initial formation of $O_2^{\bullet-}$, which later dismutates to $H_2O_2$. The Prxs-mediated $O_2^{\bullet-}$ production may be distinguished from that catalyzed by NAD(P)H-oxidase by different $K_M$ for $O_2$ and the different sensitivities of the two enzymes to inhibitors such as cyanide, azide or diphenylene

iodonium (DPI) (Bolwell et al., 1998). Indeed, Prxs activity is cyanide- or catalase-sensitive and is also favored by high concentrations of NADH (1mM). This type of activity is low without phenols or manganese, and is very active at acidic pH, whereas is relatively insensitive to DPI. On the contrary, NAD(P)H oxidase activity occurs at low concentration of NAD(P)H (< 0.2 mM), at neutral pH, and is insensitive to cyanide, catalase (CAT) and superoxide dismutase (SOD), but is inhibited by DPI (Vianello and Macri, 1991; Bindschedler et al., 2006).

Plant Prxs catalyze the single one-electron oxidation of several substrates at the expense of $H_2O_2$ (Almagro et al., 2009):

$$2RH + H_2O_2 \rightarrow 2R^{\bullet} + 2H_2O$$

Secretory Prxs usually show a molecular mass in the 30-45 kDa range and contain protohaemin IX (haem b) as prosthetic group, as well as two structural $Ca^{2+}$ ions. Crystallographic analysis and modelling studies reveal that class III plant Prxs usually contain 10-12 conserved α-helices embedding the prosthetic group, two short β-strands, and four conserved disulphide bridges (Barcelo et al., 2007).

Although many soluble intracellular and extracellular Prxs have been characterized in detail, less is known about membrane bound enzymes, in particular the Prxs of plant plasma membranes (PMs). Evidence for a PM-bound Prxs activity in higher plants has been demonstrated frequently. PMs isolated from several species and plant organs showed NAD(P)H oxidation, which was comparable to Prx activity (Møller and Berczi, 1986; Askerlund et al., 1987; De Marco et al., 1995; Vianello et al., 1990, 1997; Zancani et al., 1995; Sagi and Fluhr, 2001).

Four membrane-bound Prxs were identified in plasma membrane of maize (*Zea mays* L.) roots and the study demonstrated, for the first time, the involvement of PM-bound Prxs in oxidative stress responses. The data showed that all four membrane-bound Prxs are involved in pathogen defence with different responses to various elicitor and to methyl jasmonate and salicylic acid (Mika and Luthje, 2003; Mika et al., 2008). A first *in silico* sequence analysis of the membrane-bound class III Prxs suggested a function of these Prxs in oxidative stress at the apoplastic site of the PM (Mika et al., 2008).

# 3. ROS scavenging mechanisms

ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins and causing lipid peroxidation. The extent to which ROS accumulate is determined by the antioxidative systems, which enables organisms to maintain proteins and other cellular components in an active state for metabolism (Foyer and Noctor, 2005). Plants trigger fine-tuning of these harmful molecules level through enzymatic and non-enzymatic scavenging systems.

## *3.1 Non-enzymatic scavengers of ROS*

Low molecular mass antioxidants are redox buffers that interact with numerous cellular components. Antioxidants provide essential information on cellular redox state, and they influence gene expression associated with biotic and abiotic stress responses to maximize defence (Foyer and Noctor, 2005).

Non-enzymatic antioxidants include the major cellular redox buffers: ascorbate (ASH) and glutathione (GSH), as well as tocopherol, flavonoids, alkaloids and carotenoids. GSH is oxidized by ROS into oxidized glutathione (GSSG), ASH is oxidized to monodehydroascorbate (MDA) and dehydroascorbate (DHA). GSSG, MDA, and DHA can be reduced through the ascorbate-glutathione cycle regenerating GSH and ascorbate (Apel and Hirt, 2004).

Plants also synthesize tocopherols (vitamin E) that act as important liposoluble redox buffers. Tocopherols are a group of closely related mixed molecules (phenols and terpenes), containing a long hydrocarbon side chain and a substituted aromatic ring with a hydroxyl group that is responsible for their antioxidant activity. The α-tocopherol is associated to cellular membranes and inhibits lipid peroxidation, scavenging peroxyl radicals and preventing reaction with adjacent fatty acid side-chains or with membrane proteins (Gutteridge and Halliwell, 1994). Although α-tocopherol is considered to be a major singlet oxygen scavenger, it is also an effective scavenger of the other ROS and in this case the reduced scavenging form may be regenerated by ASH (Foyer and Noctor, 2005).

## *3.2 Enzymatic scavengers of ROS*

Enzymatic ROS scavenging mechanisms in plants include SOD, CAT, ascorbate peroxidase (APX), and glutathione peroxidase (GPX) (table1).

SODs act as the first line of defence against ROS, dismutating superoxide to $H_2O_2$. SOD is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress. The SODs remove two molecules of $O_2^{\bullet-}$ by catalyzing their dismutation, one $O_2^{\bullet-}$ being reduced to $H_2O_2$ and the other oxidized to $O_2$. It removes $O_2^{\bullet-}$ and hence decreases the risk of $OH^{\bullet}$ formation via the metal catalyzed Haber-Weiss reaction. SODs are classified by their metal cofactors into three known types: copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD), which are localized in different cellular compartments. The Mn-SOD is found in mitochondria and peroxisomes; some Cu/Zn-SOD isozymes are found in the cytosolic fractions and also in chloroplasts of higher plants. The Fe-SOD isozymes are usually associated with the chloroplast compartment (Gill and Tuteja, 2010).

CATs are tetrameric heme-containing enzymes with the potential to directly dismutate $H_2O_2$ into $H_2O$ and $O_2$; they are indispensable for ROS detoxification during stress conditions. CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert about 6 million molecules of $H_2O_2$ to $H_2O$ and $O_2$ per minute. Therefore, it is important in the removal of $H_2O_2$ generated in peroxisomes, by oxidases, during β-oxidation of fatty acids, photorespiration and purine catabolism (Gill and Tuteja, 2010).

APX is involved in scavenging of $H_2O_2$ in water-water and ASH-GSH cycles, by utilizing ASH as an electron donor. The APX family consists of at least five different isoforms including thylakoid (tAPX) and glyoxisome membrane (gmAPX) forms, as well as chloroplast stromal soluble (sAPX) and cytosolic (cAPX) forms. APX has a higher affinity for $H_2O_2$ (in the µM range) than CAT and SOD (mM range), and it may have a more crucial role in the control of ROS during stress (Gill and Tuteja, 2010).

GSH is a soluble tripeptide (γ-Glu-Cys-Gly) with a free thiol (-SH) that plays a dual role: it could react with ROS directly, but it is also a substrate or a co-factor of a transferase (GSH-Tr), a Prx (GSH-Px) or a reductase (GSH-Red). GSSG is produced by joining two GSH molecules through their –SH groups, losing two hydrogens, and forming a disulfide bridge.

The reaction is catalyzed by a GSH-Px that detoxifies $H_2O_2$ very effectively (Gutteridge and Halliwell, 1994):

$$2\ GSH + H_2O_2 \rightarrow 2\ H_2O + GSSG$$

The efficiency of the reaction depends on the availability of intracellular GSH and on the concomitant ability of the cell to reduce GSSG via the NAD(P)H-dependent GSH reductase activity (Gutteridge and Halliwell, 1994):

$$GSSG + NAD(P)H + H^+ \rightarrow NAD(P)^+ + 2GSH$$

| ENZYMATIC ANTIOXIDANTS | ENZYME CODE | REACTIONS CATALYZED |
|---|---|---|
| Superoxide dismutase (SOD) | EC 1.15.1.1 | $O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow 2H_2O_2 + O_2$ |
| Catalase (CAT) | EC 1.11.1.6 | $H_2O_2 \rightarrow H_2O + {}^1/_2O_2$ |
| Ascorbate peroxidase (APX) | EC 1.11.1.11 | $H_2O_2 + AA \rightarrow 2H_2O + DHA$ |
| Guaicol peroxidase (GPX) | EC 1.11.1.7 | $H_2O_2 + GSH \rightarrow H_2O + GSSG$ |
| Monodehydroascorbate reductase (MDHAR) | EC 1.6.5.4 | $MDHA + NAD(P)H \rightarrow AA + NAD(P)^+$ |
| Dehydroascorbate reductase (DHAR) | EC 1.8.5.1 | $DHA + 2GSH \rightarrow AA + GSSG$ |
| Glutathione reductase (GR) | EC 1.6.4.2 | $GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$ |

**Table 1**. Major ROS scavenging antioxidant enzymes. In the first column are listed the enzymes involved in the antioxidant reactions; in the second column, the respective enzyme code and in the third column the reactions catalyzed by each enzyme. AA: ascorbate; DHA: dehydroascorbate; MDHA: monodehydroascorbate; GSH: reduced glutathione; GSSG: oxidized glutathione.

# 4. Responses of plants to stress

Plants are frequently exposed to a variety of external conditions able to affect their growth, development and productivity. Their ability to adapt and live in a changing environment depends on the activation of mechanisms to withstand biotic and abiotic stresses.

Abiotic stress arises when environment deviates from optimum conditions and, consequently, cellular functions are subject to alterations (Jaspers and Kangasjarvi, 2010). Many conditions could lead to abiotic stress, ranging from drought, salinity, extreme temperature, excess light, heavy metals, ultra-violet radiation, ozone, hypoxia, and nutrient deficiency (Apel and Hirt, 2004). Biotic stress is caused by direct interaction of plant with living organisms, e.g. viruses, bacteria, fungi, herbivores etc.

Oxidative stress is a common condition during biotic and abiotic stress, being ROS generation a key process able to regulate both abiotic stress tolerance and disease resistance (Fujita et al., 2006).

## *4.1 Salicylate pathway*

As previously mentioned, ROS can trigger signalling pathways during various stresses. In plant defence against pathogens, salicylate (SA) has long been known to play a central role. SA levels increase in plant tissue following pathogen infection and exogenous application of SA results in enhanced resistance to a broad range of pathogens (Ryals et al., 1996). Genetic studies have shown that SA is required for the rapid activation of defence responses that are mediated by several resistance genes, for the induction of local defences that contrast the growth of virulent pathogens, and for the establishment of systemic acquired resistance (SAR) (Chen et al., 1993). SAR is a state of heightened defence that is activated throughout the plant, following primary infection by pathogens that elicit tissue damage at the site of infection. Several pathogenesis-related (PR) genes, whose expression is SA-dependent, are common reporters of SA-dependent defences. In this context, a great number of class III plant Prxs are generally induced by SA (Almagro et al., 2009). The attained state of resistance is long-lasting and effective against a broad spectrum of pathogens, including pathogenic bacteria, fungi, oomycetes and viruses (Durrant and Dong, 2004).

To date, two distinct pathways for the biosynthesis of SA in plants have been described (fig.3). The first involves phenylalanine, *trans*-cynnamic acid, benzoic acid, and/or *ortho*-coumaric acid as precursors for SA synthesis in plants. Production of SA, starting from

phenylalanine, occurs in the cytoplasm of a variety of plants such as tobacco (*Nicotiana tabacum*) and cucumber (*Cucumis sativus*) (Kawano et al., 2004). This pathway shares its precursors with the second, phenylpropanoid pathway, being phenylalanine ammonia lyase (PAL) one of the key enzymes. In plants, an array of compounds- often found in chemical and physical defence mechanisms against invasion by pathogens- are produced from *trans*-cinnamic acid, the first metabolite of the phenylpropanoid pathway (Kawano et al., 2004). The compounds produced via this pathway include lignin, flavonoids, phenolics, phytoalexins and SA (Kawano and Furuichi, 2007).

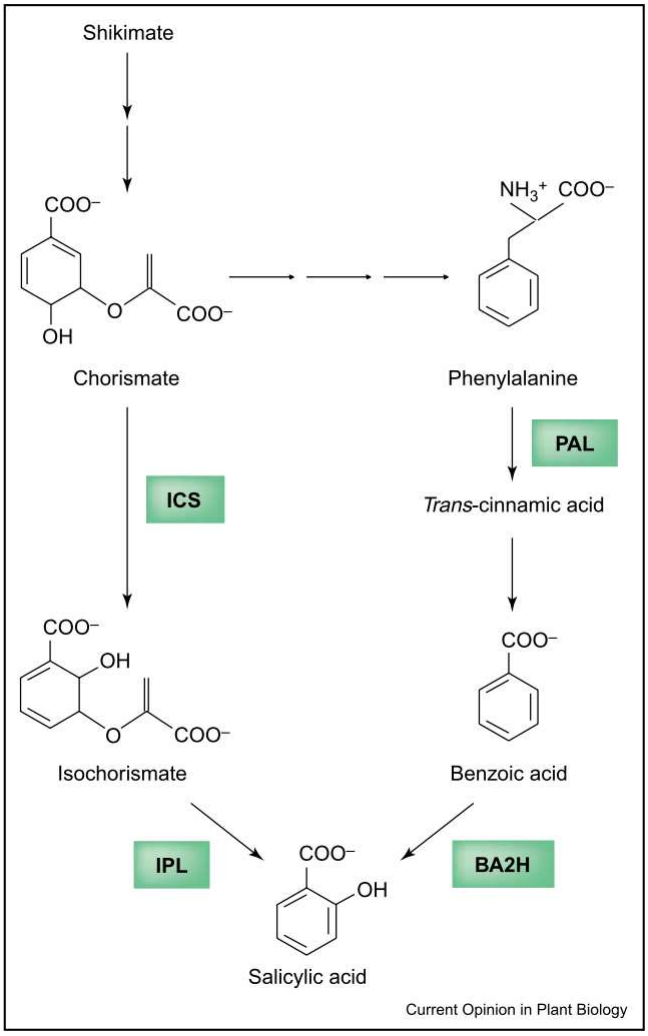


**Fig. 3** Proposed pathway for SA biosynthesis in plants (*from Shah, 2001*). ICS: isochorismate synthase; IPL: isochorismate pyruvate lyase; PAL: phenylalanine ammonya lyase; BA2H: benzoic acid 2-hydroxylase.

Recently, it has been observed that plants, as well as some bacteria, can synthesize SA from chorismic acid and isochorismic acid, two known products of the shikimate pathways. The enzymes isochorismate synthase and isochorismate lyase are responsible for the catalysis of the two steps thad lead from chorismate to SA (Shah, 2003).

## *4.2 Oxylipin pathway*

In addition to SA, other molecules involved in defence signalling pathways have been described. Attack by necrotrophic pathogens, as well as herbivorous insects, elicits the production of a large chemically diverse set of oxygenated fatty acids (oxylipins) that can be potent regulators of defence signalling. Oxylipin are bioactive lipid derivatives that act as signalling molecules in plants, animals, fungi, as well as in several marine algal species (Thaler et al., 2012). A group of oxylipins, known as jasmonates, orchestrate a large set of defence responses. Interestingly, the signalling molecule JA and other jasmonates generate a specific signal signature depending on the type of stress. In addition to playing a key role in plant defence, JA is involved in a wide range of developmental processes in the plant, such as pollen maturation, flower and fruit development, vegetative sink and storage regulation, photosynthesis, senescence, and root growth (Beckers and Spoel, 2006).

The biosynthesis of most plant oxylipins (outlined in Fig. 4) is initiated by developmental and environmental signals that regulate the biosynthesis of various oxylipins via a pathway involving the lipase-mediated release of polyunsaturated fatty acids (PUFAs) from membrane lipids. Lipases that are localized to specific cell-types and intracellular locations may direct fatty-acid substrates into multienzyme complexes that are devoted to the synthesis of a particular oxylipin (Howe and Schilmiller, 2002). Lipoxygenases (LOXs), non-heme iron dioxygenases, add molecular oxygen to PUFAs in either 9 or 13 position of the C18 chain of linoleic and linolenic acids; the terms 9-LOX and 13-LOX are used to describe enzymes that generate predominately 13- or 9-hydroperoxy fatty acids, respectively. Plants express numerous LOX isoforms that can be distinguished by their expression pattern, subcellular location, and substrate utilization (Hildebrand et al., 1998; Schewe, 1998). Detailed biochemical analyses have shown that hydroperoxy products of LOX are metabolized to an array of oxylipins by several enzymes, including allene oxide synthase (AOS), hydroperoxide lyase (HPL), divinyl ether synthase (DES), epoxy alcohol synthase, peroxygenase, alkyl hydroperoxide reductase, and LOX itself (Howe and Schilmiller, 2002). The relative specificity of these enzymes for either 9- or 13-hydroperoxides supports the

concept that oxylipin metabolism is organized into discrete 9-LOX and 13-LOX pathways, each of which is divided into several cytochrome P450 (CYP74)-dependent sub-branches.

The AOS branch of the 13-LOX pathway transforms 13-hydroperoxy linolenic acid (13-HPOT) into the jasmonate family of compounds including JA/Methyl-JA and their metabolic precursor, as well as 12-oxo-phytodienoic acid (12-OPDA). The HPL branch of the 13-LOX pathway directs the formation of C6 aldehydes and C12 ω-keto-fatty acids. The C12 product derived from linolenic acid is the precursor of traumatin, a mitogenic compound that is implicated in wound healing (Zimmerman and Coudron, 1979). C6 aldehydes, together with their corresponding reduced alcohols, are important volatile constituents of fruits, vegetables and green leaves. Increasing evidence indicates that short-chain aldehyde products of the 13-HPL pathway also play important roles in defence against microbial pathogens and insects (Bate and Rothstein, 1998; Vancanneyt et al., 2001).

The metabolism of 9-hydroperoxy fatty acids by AOS, HPL, and DES generates a group of oxylipins that are structurally related to, but distinct from, the oxylipins derived from the 13-LOX pathway. Several recent studies indicate that the 9-LOX pathway performs an essential role in plant defence against microbial pathogens.

Oxylipin biosynthesis is regulated by several stress-induced (herbivorous and wounding, pathogen attack, osmotic shock, drought, UV light) and developmental cues (anther deiscence, pollen development, tuberization, senescence, flowering and storage) (Creelman and Mullet, 1997; Hildebrand et al., 1998; Kramell et al., 2000).

Although the underlying regulatory mechanisms responsible for changes in oxylipin formation are complex and difficult to generalize, recent evidence indicates that phospholipases (PLase), releasing fatty-acid precursors from membrane lipids, are key components of this regulation (Howe and Schilmiller, 2002).

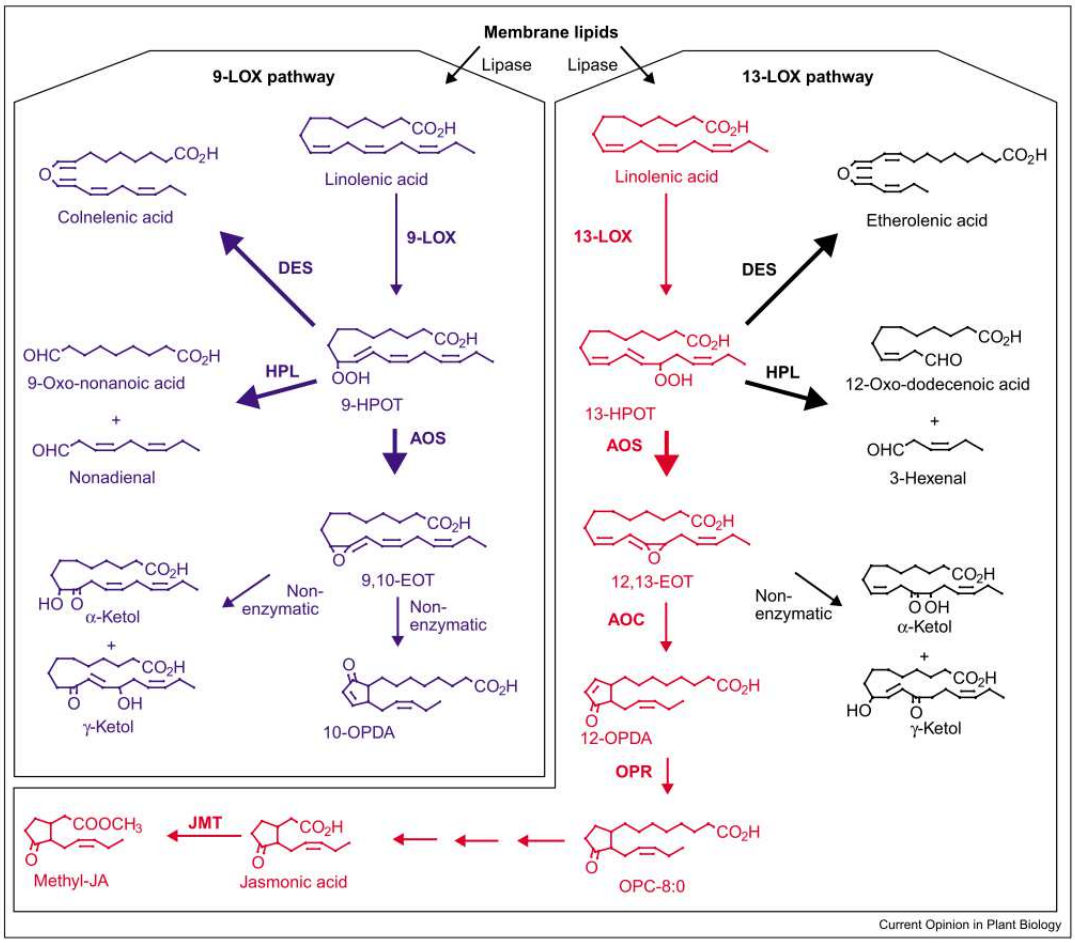


**Fig. 4** The 9-LOX and 13-LOX pathways for the metabolism of linolenic acid. 9- and 13-hydroperoxides of linolenic acid are substrates for members of the CYP74 family of cytochrome P450s (*from Howe and Schilmiller, 2002*). LOX: lipoxygenase; DES: divinyl ether synthase; HPL: hydroperoxide lyase; AOS: allene oxide synthase; AOC: allene oxide cyclase; OPR: oxo-phytodienoic acid reductase; JMT: JA carboxyl methyltransferase.

## *4.3 SA & JA cross-talk*

Plants have to balance the costs and potential benefits of investing in defence in an environment where enemy attack is variable. The inducible plant defence system can be generally divided into two branches: one effective primarily against biotrophic (feeding on living tissue) pathogens, and the other against herbivores and necrotrophic (feeding on dead tissue) pathogens (Stout et al., 2006). There are many kind of inducible defences, including morphological structures (e.g. trichomes), killing toxins (e.g.alkaloids), digestibility reducers (e.g. proteinase inhibitors) and indirect defences (e.g. extrafloral nectaries and plant volatiles) that can attract other insects that deter herbivores (Thaler et al., 2012). Several plant hormones regulate the production of downstream resistance molecules in each branch. The SA pathway is primarily induced by and effective in mediating resistance against biotrophic pathogens and

the JA pathway is primarily induced by and effective in mediating resistance against herbivores and necrotrophic pathogens (Glazebrook, 2005). This is a simplistic view of the complex repertoire of plant hormones that probably play a role in mediating inducible defences, including abscisic acid (ABA), auxin, brassinosteroids, cytokinins, ethylene (ET) and gibberellic acid. Interestingly, evidence from several distantly related plant species suggests that there can be evolutionarily conserved SA- and JA-signalling crosstalk resulting in reciprocal antagonism between the SA and JA signalling pathways (Glazebrook, 2005).

Although it can be a reciprocal antagonism, downregulation from each side of the SA and JA pathway is not identical and may not be antagonistic across plants (Thaler et al., 2012). SA is typically prioritized over JA in Arabidopsis (Leon-Reyes et al., 2010b). However, plants use ET to fine-tune defences by prioritizing JA induction over SA in response to multiple attackers (Leon-Reyes et al., 2010a). ET also modifies the effect of a key protein (NPR1; Non-expressor of pathogenesis-related genes 1) involved in SA suppression of JA. In Arabidopsis, NPR1 is necessary for expression of SA-responsive genes and for repression of JA by SA. However, when ET is present, NPR1 function is no longer required for SA suppression of JA (Leon-Reyes et al., 2010a), suggesting that ET signalling acts to suppress JA in the presence of SA, bypassing NPR1.

SA-JA antagonism has been reported in 17 plant species, including 11 crop plants and six wild species (Thaler et al., 2012). Orthologs of genes involved in the SA-JA antagonism, based on reciprocal blastp searches using Arabidopsis proteins as subjects, including NPR1, WRKY70 (WRKY dna-binding protein70), GRX480 (Glutaredoxin 480), ERF1 (Ethylene response factor 1), MYC2 (Jasmonate insensitive 1, JIN1), ORA59 (Octadecanoid-responsive arabidopsis AP2/ERF 59), JAZ1-JAZ3 (Jasmonate zim-domain), are predicted to have been present in the first land plants, after their lineage split with green algae. This suggests that many regulatory features of SA-JA crosstalk have diverse and potentially ancient roles in the cell. An orthologous of the canonical cross-talk regulator NPR1 was probably present in the ancestor of all land plants, indicating that the potential for this gene to mediate SA-JA antagonism exists in all species in which the antagonism has been found. NPR1 exhibits unique roles in SA-JA crosstalk in different plant species. Functional data, based on gene expression and/or other studies, show that NPR1 modulates SA-JA antagonism in rice, Arabidopsis and tomato, suggesting that this subfunction for NPR1 may have ancient origins in the common ancestor of monocots and eudicots. Together, the role of NPR1, WRKY and JAZ genes in regulating SA-JA and SA-JA-ET crosstalk from rice to eudicots suggests a

generally conserved core genetic architecture of defence signalling in flowering plants (Thaler et al., 2012).

SA induction frequently suppresses JA induction. Nevertheless, in seven species it has been observed that JA responses were associated with the suppression of SA induction (Seo et al., 1997; Liu et al., 2008). However, very few studies examined SA-JA antagonism in a field setting (Thaler et al., 1999; Rayapuram and Baldwin, 2007).

In some cases, SA and JA pathways are each upregulated by one attacker species, but their induction is not simultaneous. For example, following infection by *Fusarium* spp., a hemibiotrophic fungal pathogen, SA and JA pathways are both induced after infection, but SA acts in order to establish the resistance early on, and JA is important in facilitating resistance during later time points (Ding et al., 2011). Thus, the same pathogen induces the SA and JA pathways but the responses are temporally disconnected. Antagonism is highly context-dependent, both in terms of what is used to elicit SA and JA, the timing of the elicitation, and possibly with respect to genetic variation underlying the antagonism (Thaler et al., 2012).

## *4.5 A case of study: recovery in apple plants*

Apple proliferation (AP) is one of the most economically important phytoplasmal diseases in Europe. AP occurs in most European countries where apple is grown, but it is of economic importance mainly in a zone across the middle Europe. The southern extension of this zone reaches to Southern France, the Po valley of Italy, the Balkan states and Northern Greece, while recently the disease has been identified in Turkey (Sertkaya et al., 2008).

Causal agent of the disease is a phytoplasma mainly (or only) spread by psyllids (*Psyllidae*) (Frisinghelli et al., 2000). Recently, the AP phytoplasma has been delineated taxonomically under the provisional status "*Candidatus*" for uncultured bacteria as "*Candidatus* Phytoplasma mali". Based on sequence analysis of 16S rDNA, "*Ca*. P. mali"is assigned to the AP or 16SrX group in the phytoplasma phylogenetic clade (Seemuller and Schneider, 2004).

AP induces specific and nonspecific symptoms on shoots, leaves, fruits, and roots. The first indication of disease is foliar reddening in late summer to mid fall, which is in contrast to the normally yellow fall coloration of healthy trees. In July or August of the following year, symptomatic trees may form witches' broom through the suppression of apical dominance and by the growth of normally dormant axillary buds in the upper part of vigorous shoots. The

resulting secondary shoots are steeply erect and differ from normally wide-angled growth of lateral buds. Other symptoms, characterizing AP, are formation of smaller leaves with enlarged stipules, leaf rosette and abnormal flowers (Hadidi et al., 2011). Leaves are light green or chlorotic and are often smaller and more susceptible to powdery mildew than the normal ones. The vigour of young trees is severely reduced and their root system is poorly developed. Trees, infected at a later stage and with a well-developed root system, when become infected suffer less from the disease and show a better recovery (Seemuller, 1990). AP considerably reduces yield and fruit quality. While fruit number is usually not affected, fruit weight is often reduced by 30-60 %, the taste is poor, with the result that as much as 80% of the fruits are unmarketable (Hadidi et al., 2011).

Apple trees affected by AP can spontaneously recover from the disease, which implies the disappearance of the symptoms and pathogens from the canopy, but not from the roots, where phytoplasma persist viable and infectious (Osler et al., 1999). Recently, it has been observed that in apple (Musetti et al., 2004), apricot (Musetti et al., 2005) and grapevine (Musetti et al., 2007), the recovery is linked to an overproduction of $H_2O_2$ in the phloem tissues. This implies the involvement of ROS, in particular $H_2O_2$, during the defence response to this biotic stress.

In addition to high level of $H_2O_2$ in the phloematic cells, recovery has been associated to lower antioxidant activity (Musetti et al., 2007), deposition of callose and phloem-proteins (Musetti et al., 2010), high concentration of phloematic $Ca^{2+}$ in the leaves (Musetti et al., 2010).

In spite of all experimental evidence above described, many physiological aspects of recovery are still poorly understood. In particular, it has not been found a direct link between the observed physiological responses to the infection and the signalling and metabolic pathways responsible for their expression. Therefore further investigations are needed in order to clarify the biochemical patterns associated to this phenomenon.

## 5. Intermediate and recalcitrant seeds

Depending on desiccation tolerance, seeds are generally subdivided into three groups: orthodox, intermediate and recalcitrant (Ellis et al., 1990)

Moisture content of the major part of mature seeds, orthodox seeds, is around 2-5 % and sometimes they can be dried even down to 0.5 % without any loss of viability. Thus, orthodox

seeds can tolerate drying to low water content, and their storage life span increases along with the decrease in both moisture content and temperature, in a measurable and predictable way.

On the contrary, in some species mature seeds do not dry out on the mother plant, and their moisture content is very high when they are dispersed. In addition, if seed moisture content drops below 30-65 %, depending on the species, viability is lost; therefore they are defined as recalcitrant seeds (Baskin and Baskin, 1998). Roberts (1973) introduced the term "recalcitrant" to describe seeds that undergo little or no drying, and remain sensitive to desiccation both during development and after dissemination. Such seeds are shed in a hydrated condition, and the water content can generally vary from 30 to 80 % on a wet mass basis and retain an appreciable metabolic activity (Roberts, 1973). Therefore, recalcitrant seeds cannot be stored in a desiccated condition and are not suitable to long-term storage (Berjak and Pammenter, 1997). Generally, recalcitrant seeds need to be sown or processed immediately after collection from the mother plant, otherwise they may die. Thus, the developmental status of the seeds is modified after they are collected from the plant (Berjak et al., 1989).

A further class, the "intermediate" seeds, has been described by Ellis and colleagues (1990); these seeds are more tolerant to desiccation than recalcitrant seeds, but not to the extent of orthodox seeds, and many of them lose viability more rapidly at low temperatures. Using coffee plant as a model, Ellis et al. (1990) defined the "intermediate" category as the seeds that display the following two main characteristics regarding their level of desiccation tolerance and storage behaviour: i) seeds that are able to withstand considerable drying (relative humidity, RH of 30-40 %) in comparison with recalcitrant seeds, but ii) which cannot tolerate extreme water loss as in the case of orthodox seeds; in contrast with orthodox seeds, lowering the storage temperature decreases seed longevity at low water contents (RH < 50 %). Therefore, intermediate seeds cannot be stored under conventional gene bank conditions (FAO/IPGRI 1994), and for intermediate seeds species cryopreservation is the only technique available for long-term germplasm conservation (Dussert et al., 2001, 2003). These seeds do not achieve dehydration and remain metabolically active (Pasquini et al., 2012). Since intermediate seeds do not reach maturation drying and the water content inside the ripe fruit is around 50 % (Wintgens, 2004), they do not undergo a resting period and seed germination is initiated while the fruit is still in the final stages of development. This germination is associated with an activation of the respiration (Berjak and Pammenter, 2008) and to an unpaired antioxidant system, whose consequences are the generation and over-accumulation of both toxic free radicals and harmful volatiles, such as ethanol and

acetaldehyde (Finch-Savage et al., 1993). In the case of coffee beans, during postharvest treatment significant metabolism occurs within the seeds (Bytof et al., 2005), as it will be widely discussed later (par. 6.1).

## *5.1 Lipid metabolism during germination*

Seed germination is a critical developmental stage in the life cycle of seed plants, because it is a complex and multistage process. Subsequent seedling growth needs large amounts of energy and nutrients, which can be provided only by seed reserves, since the germinating seed lacks a mineral uptake system and a photosynthetic apparatus (Bewley, 1997). During germination, the quiescent embryonic cells shift into a metabolically active state in which complex biochemical and physiological changes occur (Yang et al., 2009).

In oilseeds the process of germination is characterized by the mobilization of the storage lipids, which serve as major carbon source for growth of seedlings. Triacylglycerols (TAGs) are accumulated in specialized structures named oil bodies or oleosomes. They are usually 0.2-2 µm in diameter, providing a high surface to volume ratio, which is believed to facilitate the rapid conversion of TAGs into free fatty acids (FFAs) via lipase-mediated hydrolysis at the oil body surface (Huang, 1996). Oil bodies are composed of a hydrophobic core of TAGs surrounded by a monolayer of phospholipids (PLs) coated with specific proteins (Murphy, 2001). The most abundant of these proteins are the oleosins, usually present as two or more highly conserved isoforms (Tzen et al., 1990). They are thought to help stabilizing the lipid body during seed maturation, desiccation and germination by preventing the coalescence of the organelles (Cummins et al., 1993; Leprince et al., 1998).

Knowledge about early events of lipid mobilization during germination is still scarce. Until now evidence has been found for proteolytic degradation of the oleosins (Matsui et al., 1999; Vandana and Bhatla, 2006). However, data on the direct degradation of the PL monolayer by TAG lipase has been reported only for lipid bodies from almond (Beisson et al., 2001). Degradation of lipid body membrane by a patatin-like phospholipase has been reported for cucumber and sunflower (Noll et al., 2000; Gupta and Bhatla, 2007). Studies on cucumber seedling demonstrated that during early stage of lipid mobilization a patatin-like protein, exhibiting PLase activity, may be present in lipid bodies (May et al., 1998). This protein (CsPAT) seems to be exclusively confined in lipid bodies (May et al., 1998). When CsPAT reaches its maximal expression level, the PL monolayer of cucumber lipid bodies contains a hole of 80 nm in width and 2.45 nm in depth, sufficiently large to provide access to 100 kDa

enzymes such TAG lipase (Noll et al., 2000). These findings suggest the involvement of a specific lipid body PLase in the mobilization of storage lipids by facilitating the decomposition of oil body membranes (Rudolph et al., 2011).

Efficient oil breakdown is essential for successful seedling establishment (Yang et al., 2009). Therefore, seed germination is triggered by the induction of many specialized biochemical pathways in different subcellular locations (Graham, 2008). Lipases start the oil breakdown by hydrolyzing TAGs to produce FFAs and glycerol (Huang, 1992). The FFAs then enter single membrane bound glyoxysomes, where β-oxidation and part of the glyoxylate cycle occurs (Cooper and Beevers, 1969a, 1969b). Glyoxysomes are metabolically distinct from peroxisomes, since they contain two enzymes that are unique to the glyoxylate cycle, malate synthase and isocitrate lyase (Graham, 2008). β-oxidation converts FFAs to acetyl-CoA and subsequently, via the glyoxylate cycle, two molecules of acetyl CoA generate one molecule of succinate in the glyoxysome. The succinate is transferred to the mitochondria and converted into malate by some reactions of the citric cycle. Then, the malate is released from mitochondria to the citosol where it is converted into phosphoenolpyruvate, a precursor for the synthesis of hexoses by the gluconeogenesis pathway. The intermediates formed can be used in the pentose phosphate pathway for the biosynthesis of other compounds such as RNA and proteins. The macromolecules from gluconeogenesis and other processes can be transported to the growing embryo by the activity of plasmodesmata-associated proteins (Yang et al., 2009). Four carbon compounds, from the glyoxylate cycle, can be converted into hexoses by gluconeogenesis and, subsequently, used for cell wall biosynthesis or converted into sucrose for transport to the growing seedling tissue (Canvin and Beevers, 1961).

Oil bodies and glyoxysomes are in close proximity, as suggested by a number of electron micrographic studies (Wanner and Theimer, 1978; Hayashi et al., 2001), and invagination has been proposed as a direct mechanism to transport lipids from the oil bodies into glyoxysomes during FAs β-oxidation (Hayashi et al., 2001). Biochemical data demonstrated that neutral lipids are transferred directly from the oil body into the glyoxysomal membrane of cotton (*Gossypium hirsutum*) seedling, supporting the relevance of physical contact between the organelles (Chapman and Trelease, 1991).

Yang et al. (2009) studied oil mobilization during seed germination and post-germination development of *Jatropha curcas* with a proteomic approach in combination with an ultrastructural observation of the endosperm. The oil content in endosperm displays a strong decrease after 60 h of imbibition, indicating that the mobilized oil in endosperm is transferred to the embryo for developing seedlings. These results imply that large-scale lipid

mobilization is initiated during germination, and the mobilized oil is used in post-germination for establishment of seedlings (Yang et al., 2009).

## *5.2 Oxidative stress: oxylipin pathway associated to lipase and phospholipase activity*

TAG lipases (E.C.3.1.1.3) hydrolyze TAGs at the oil/water interface to yield FFAs and glycerol. They have been characterized from numerous organisms and belong to the super-family of α/β fold hydrolases characterized by a catalytic triad composed of Ser, His, and Asp or Glu (Ollis et al., 1992). Lipase activities are typically membrane-associated and can be found in oil bodies, glyoxysomes, or microsomal fractions of seed extracts, depending on the species (Huang, 1983, Mukherjee, 1994). Lipases have been purified from the seeds of a number of plants: maize (*Zea mays*) (Lin and Huang, 1984), castor bean (*Ricinus communis*) (Maeshima and Beevers, 1985; Fuchs et al., 1996), oilseed rape (*Brassica napus*) (Fuchs and Hansen, 1994) and ironweed (*Vernonia galamensis*) (Ncube et al., 1995). More recently, several genes encoding proteins with TAG lipase activity have been cloned from castor bean, Arabidopsis and tomato (*Solanum lycopersicum*) (Eastmond, 2004, Matsui et al., 2004). All these lipases are typical α/β hydrolases and are able to hydrolize TAGs, but not PLs or galactolipids (Quettier and Eastmond, 2009). Generally, low lipase activity can be detected in imbibed seeds before germination, and the activity increases significantly following germination, coinciding with TAGs mobilization (El-Kouhen et al., 2005).

Important advances in the search of the TAG lipases were achieved via the use of an Arabidopsis seedling mutant screen (Eastmond, 2006). Disruption of genes involved in TAGs utilization in Arabidopsis leads to postgerminative growth arrest and this phenotype could be rescued by the addition of an alternative carbon source, such as sucrose, in the medium (Hayashi et al., 2001). The sucrose rescue method allowed to screen mutagenized seeds and six new loci were isolated, three of which are impaired in TAGs hydrolysis upon germination (Eastmond, 2006). Cloning of the first mutant locus, called sugar dependent1 (*sdp1*), revealed that it encodes a patatin-like TAG lipase, that contains a patatin-like serine esterase domain, previously described in animal and yeast TAG lipases, which are required for TAGs breakdown (Athenstaedt and Daum, 2003; Zimmermann et al., 2004; Gronke et al., 2005). SDP1 is active against long chain TAGs in vitro, and green fluorescent protein fusions show that it is associated with oil bodies during germination. SDP1 homologues are found in a wide

range of plant species (Eastmond, 2006) and represent a class of TAG lipases conserved across eukaryotes for the hydrolysis of TAGs from oil bodies (Graham, 2008).

PLs provide the backbone for biomembranes, serve as rich sources of signalling messengers, and occupy essential functions in lipid metabolism. The activities of PLase not only affect the structure and stability of cellular membranes, but also regulate many cellular functions (Wang, 2001). Activation of PLase is often an initial step in generating lipid and lipid-derived second messengers (Wang, 2001).

PLase constitute a wide series of enzymes that can be classified into PLase D (PLD), C (PLC), A2 ($PLA_2$), A1 ($PLA_1$), and B (PLB), according to their sites of hydrolysis on PLs (Fig. 5). Each class is divided further into subfamilies based on sequences, biochemical properties, or a combination of both (Wang, 2001).

Based on their catalytic activity at the specific positions on a membrane glycerophospholipid, PLA superfamily has been divided into $PLA_1$ and $PLA_2$ subtypes. $PLA_1$ catalyzes the hydrolysis at sn-1 position of a PL whereas $PLA_2$ acts on sn-2 position (Singh et al., 2012). Plant $PLA_1$ members are characterized by the presence of a highly conserved GXSXG motif, by Ser, Asp and His in the catalytic centre, and a molecular weight of 45–50 kDa (Matsui et al., 2004; Chen et al., 2011). In plants, different PLA members have been characterized and reported to be involved in seed (May et al., 1998) and root development (Rietz et al., 2010), wounding and pathogen attack (Laxalt and Munnik, 2002), hyperosmotic stress (Munnik and Meijer, 2001), cold and high salinity stress (Narusaka et al., 2003).

Products generated in the reaction, such as FFAs and lysophospholipids, lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) are biologically active compounds, being involved in several cellular signalling pathways (Ryu, 2004). FFAs, including linolenic acid, can subsequently be converted, by downstream metabolic enzymes, into bioactive oxylipin compounds such as JA and its derivatives (Ryu, 2004).

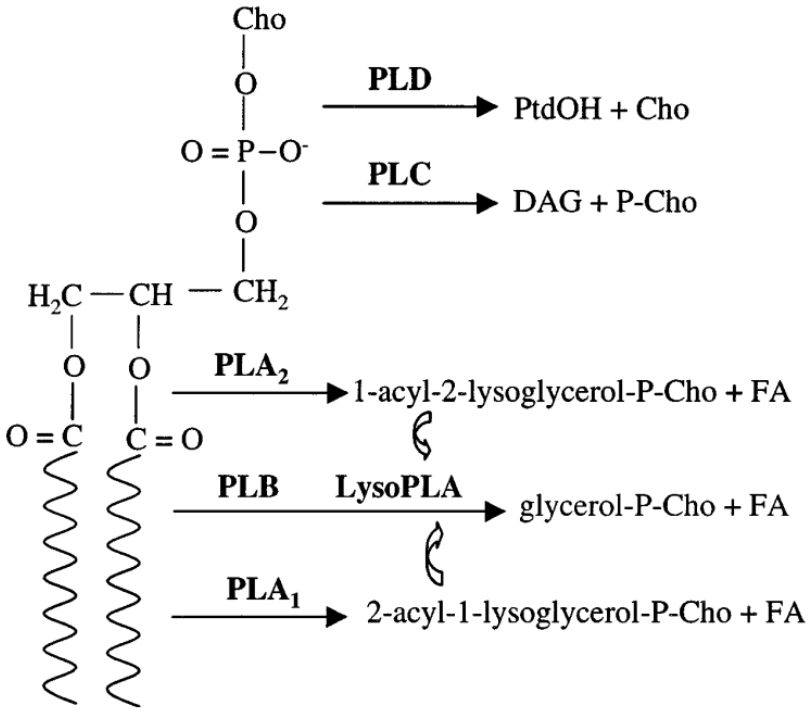


**Fig. 5** Phospholipase and relative site of hydrolisis (*from Wang, 2001*). PLA: phospholipase A; PLB: phospholipase B; PLC: phospholipase C; PLD: phospholipase D. Cho: choline; P-Cho: phosphocholine; FA, fatty acid; PtdOH: phosphatidic acid; DAG: diacylglycerol.

PLB removes the last fatty acid from lysophospholipid (Wang, 2001) (Fig. 5). Lysophospholipids in plants are produced in response to stress cues, and modulate a number of enzyme activities (Scherer, 1995; Chapman, 1998; Munnik et al., 1998; Paul et al., 1998).

PLC hydrolizes the glycerophosphate ester linkage of PLs to diacylglycerol and phosphorylated head groups (Fig. 5). PLC is involved in plant response to variuos stimuli, like osmotic stress, abscisic acid, light, gravity, pathogen attack, and pollination (Shi et al., 1995; Chapman, 1998; Munnik et al., 1998; Perera et al., 1999; Staxen et al., 1999).

PLD catalyzes the hydrolysis of structural PLs, producing phosphatidic acid and a free headgroup (Fig. 5) (Bargmann and Munnik, 2006). It is involved in seed germination, ageing, senescence and under a broad spectrum of stress conditions, like freezing, drought, wounding, pathogen infection, nutrient deficiency and air pollution (Young et al., 1996; Chapman, 1998; Frank et al., 2000; Wang, 2000).

Fatty acid released by lipases and PLases can enter β-oxidation cycle, but can also be metabolized by LOX, that converts polyunsaturated fatty acids into their corresponding hydroperoxy derivatives (Weichert et al., 2002), more reactive forms of fatty acids. Linoleic acid (LA) (18:2) is a major storage fatty acid in many oilseeds. With LA as substrate, either

(9S)- or (13S)-hydroperoxy octadecadienoic acids may be formed, while with α-linolenic acid (LeA), as substrate, either (9S)- or (13S)-hydroperoxy octadecatrienoic acids are produced. These C18 hydroperoxides can be further converted by at least seven different enzyme families belonging to the oxylipin pathway (Feussner and Wasternack, 2002), as above described (par. 4.2). A main branch metabolizing LOX-derived products is initiated with the fatty acid HPL reaction leading to fragmentation into C6 or C9 aldehydes and C12 or C9 ω-oxo fatty acids (Weichert et al., 2002), and into several other cytotoxic products including saturated aldehydes (hexanal) and unsaturated aldehydes, like 4-hydroxy-2-nonenal or malondialdehyde (Zacheo et al., 2000). Moreover, lipid peroxidation also produces exceedingly reactive free radical intermediates such as conjugated dienes that have considerable potential to deteriorate or reduce longevity of seeds (Khan et al., 1996; DeLong et al., 2002; Li et al., 2010). Products deriving from 13-HPL, hexanal and hexenal, are involved in plant signalling as well as defence, and are major contributors to the aroma of many fruits, vegetables, and green leaves (Hatanaka, 1993). Aldehydes formed by HPL activity can be further metabolized to their corresponding alcohols (Salas and Sanchez, 1999).

# 6. A case of study: oxidative stress in coffee seed during storage and Germination

The coffee plant is a shrub characterized by a single or multiple vertical stems carrying primal horizontal branches at each internode, forming secondary horizontal branches. The *Coffea* genus belongs to the *Rubiaceae* family and includes about a hundred species of which the most important are *Coffea arabica* and *Coffea canephora*, Robusta variety. The origin of *C. arabica* is in Ethiopia (Abyssinia), in high plateaux areas at altitudes between 1300 and 2000 m above sea levels (Charrier et al., 2009).

The coffee plant, usually after three years of development, could blossom and produce the fruit, commonly named “cherry” or “coffee berry”. The fruit consists of a pericarp, red or yellow at ripening, usually including two seeds. The seed is elliptical, composed of a hard endosperm that contains the embryo, which is enfolded into two casings: the parchment on the outside, and the silverskin just under the parchment. The endosperm is composed solely of uniform living reserve cells, and endosperm cells undergo programmed cell death only after reserves have been mobilized during seedling growth (Joet et al., 2009).

The embryo is 3-4 mm long and is composed of the hypocotyl (embryo axis) and two cotyledons. The coffee endosperm (approx. 99% of the mature seed mass) contains seed storage reserves necessary for seedling growth. These susbstances are of great importance also for the food industry, being important precursors of coffee aromas during roasting. The major storage compounds of mature seeds of *C. arabica* are cell wall polysaccharides (CWP, 48–60% DM), mainly galactomannans and arabinogalactan proteins (Redgwell et al., 2002, 2003), lipids (13–17% DM), mainly as TAGs (Speer and Kölling-Speer, 2006), proteins (11–15% DM), (Clifford, 1987), sucrose (7–11% DM), (Campa et al., 2004) and chlorogenic acids (CGA) (5–8% DM, (Farah and Donangelo, 2006). Each of these major storage compounds plays several crucial roles in the complex roasting chemistry (Flament, 2002). For example, proteins and amino acids are essential for the conversion of reducing sugars (which result from the degradation of sucrose and CWP) into aroma precursors through Maillard reactions (DeMaria et al., 1996). TAGs are the major aroma carrier in the roasted bean (Petracco, 2005) and their fatty acid composition determines the generation of thermally induced oxidation products, in particular aldehydes, which react readily with Maillard intermediates, giving rise to additional aroma compounds (Flament, 2002). Another peculiar feature of coffee seeds is the fact that they accumulate considerable amounts of CGA, which are mobilized during germination for seedling lignin synthesis (Aerts and Baumann, 1994).

The lipid content of green Arabica coffee beans averages some 15%, most of which as coffee oil, is located in the endosperm of green coffee beans (Wilson et al., 1997); only a small amount, the coffee wax, is located on the outer layer of the bean. Coffee oil is composed mainly of TAGs with fatty acids (table 2) similar in proportions to those found in common edible vegetable oils. The relatively large unsaponifiable fraction is rich in diterpenes of the kaurane family, mainly cafestol, kahweol and 16-O-methylcafestol, which have been receiving more attention in recent years, due to their different physiological effects (Speer and Kölling-Speer, 2006). Fatty acid for the most part are present in a combined state; most are esterified with glycerol in TAGs, about 20 % are esterified with diterpenes, while a small proportion is present in the sterol esters (Speer and Kölling-Speer, 2006).

Oliveira and coworkers (2006) analyzed the fatty acid content of *C. arabica*. The main fatty acids found by these authors were linoleic (44 %) and palmitic acid (34 %), followed by oleic (9 %) and stearic acid in moderate quantities (7 %) and small amounts of arachidonic (3 %), linolenic (1.5 %), behenic (0.7 %) and eicosanoic acid (0.3 %). The same authors studied the differences in fatty acid composition between defective and non-defective coffee beans but no significant differences were found.

| Common name | Lipid name | Folstar (1975) from dewaxed green beans | Speer (1993) | Speer (1993) |
|---|---|---|---|---|
| | | | Robusta (n=9) | Arabica (n=4) |
| Myristic acid | $C_{14:0}$ | 0.2 | traces | traces |
| Pentadecanoic acid | $C_{15:0}$ | | traces | traces |
| Palmitic acid | $C_{16:0}$ | 33.3 | 27.2-32.1 | 26.6-27.8 |
| Palmitoleic acid | $C_{16:1}$ | | traces | traces |
| Margaric acid | $C_{17:0}$ | | traces | traces |
| Stearic acid | $C_{18:0}$ | 7.3 | 5.8-7.2 | 5.6-6.3 |
| Oleic acid | $C_{18:1}$ | 6.6 | 9.7-14.2 | 6.7-8.2 |
| Linoleic acid | $C_{18:2}$ | 47.7 | 43.9-49.3 | 52.2-54.3 |
| Alpha Linoleic acid | $C_{18:3}$ | 1.7 | 0.9-1.4 | 2.2-2.6 |
| Nonadecylic acid | $C_{19:0}$ | | traces | traces |
| Arachidic acid | $C_{20:0}$ | 2.5 | 2.7-4.3 | 2.6-2.8 |
| Eicosenoic acid | $C_{20:1}$ | | 0.2-0.3 | traces-0.3 |
| Heneicosanoic acid | $C_{21:0}$ | | traces | traces |
| Behenic acid | $C_{22:0}$ | 0.5 | 0.3-0.8 | 0.5-0.6 |
| Tricosylic acid | $C_{23:0}$ | | traces | traces |
| Lignoceric acid | $C_{24:0}$ | traces | 0.3-0.4 | 0.2-0.4 |

**Table 2**. Fatty acids in triacylglycerols of green coffee beans (%) (*from Speer and Kölling-Speer, 2006*, modified).

## *6.1 Green coffee processing*

Traditionally, green coffee is produced either by wet or dry processing methods. In wet processing, ripe coffee cherries are mechanically depulped and the mucilaginous residues of the pulp are degraded by fermentation. The resulting beans are still covered by the endocarp, the parchment. Then, the seeds are dried, conditioned and subsequently hulled. In contrast, in dry processing, the entire coffee fruits are dried without removal of the pulp and then hulled mechanically. In both cases, the freshly-processed coffee beans remain viable (Huxley, 1964; Cléves, 1998) and exhibit active metabolic processes (Selmar et al., 2006). In the course of wet processing, the fruit flesh (pulp) is removed, permitting initiation of seed germination. In contrast, during dry processing the pulp remains around the seeds; germination should not occur until endogenous "unlocking" occurs. During the course of storage, the coffee seeds die (Huxley, 1964; Couturon, 1980). Precocious germination within the fruit is most likely prevented by the joint effects of abscisic acid and the osmotic potential of the fruit flesh (Bewley and Black, 1994). Thus, the extent and progression of germination should be different in coffee processed by these two methods (Selmar et al., 2006). Substantial features of a seed in state of germination are an increasing rate of respiration and the mobilization of storage compounds. These physiological processes must have an impact on the concentration of aroma precursors in green coffee beans and thus on coffee quality (Bytof et al., 2000).

Depending on the processing method applied, the composition of low molecular weight carbohydrates (Knopp et al., 2006), amino acids (Selmar et al., 2002; Bytof et al., 2005) and the level of γ-aminobutyric acid (GABA) (Bytof et al., 2005; Knopp et al., 2006), a stress metabolite, can differ in green coffee. Both free amino acids and free sugars are essential precursors of the coffee flavour (Bytof et al., 2000; Bradbury, 2001; Homma, 2001). Flavour differences among the two differently processed green coffee must be the consequence of metabolic processes in the coffee beans during post-harvest processing (Selmar et al., 2006). Selmar et al. (2006) argued that, since seed maturation is already accomplished in ripe coffee cherries (Wormer, 1964), the most likely metabolic events in living seeds during the course of processing should be related to germination. In fact, they demonstrated that coffee seeds undergo germination-related metabolism during processing, either dry or wet, by analyzing the expression of the key enzyme of the glyoxylate cycle, isocitrate lyase, to determine the transition from late embryogenesis to germination, and expression of β-tubulin, a marker of cell division. During wet processing, both isocitrate lyase and β-tubulin expression increased significantly within the first two days of treatments, while during dry processing both markers gave stronger signals later, after 4 to 5 days after the start of the treatment. Germination-

associated metabolism during post-harvest of green coffee not only resulted active, but also differently regulated, according to the processing method (Selmar et al., 2006).

## *6.2 Green coffee storage*

Storage is a critical stage in the processing of any agricultural commodity and, in the case of coffee, the aim is to preserve the intrinsic quality of the beans as long as possible (Rojas, 2009). Coffee market is characterized by an imbalance between demand and supply, since coffee production is seasonal; those aspects lead to the necessity of storing the coffee for long periods of time, often just to wait for a better selling price. One year is generally the accepted time for green coffee storage under normal conditions, but in some countries, like Brazil, coffee could be stored for more than four years (Rojas, 2009). Usually, commercial coffee is stored up to three years (Scheidig and Schieberle, 2006).

The main features involved in storage coffee seeds are the limit to which they can be dried and the interaction of temperature and water content on seed survival (Eira et al., 2006). Coffee seeds do not require a period of dormancy and, consequently, for propagation purposes they should be sown as soon as possible after ripening, when their moisture content is over 50 %. At this stage, their germination rate is more than 90 %, but seed viability decreases rapidly after 6 months when stored at ambient temperature. If seeds have to be stored, they should maintain the parchment and have to be dried slowly at a low temperature (not above 40 °C), lowering their moisture content to 12-13 % (Rojas, 2009).

During their shelf life, the beans retain all the characteristics and activities including respiration and transpiration. For respiration, the seed reserve substances, such as starch, carbohydrates, lipids and proteins are consumed by enzymatic processes to generate $CO_2$ and water in exothermic reactions. The impact of respiration on bean deterioration is noteworthy: every 24 h, 100 g of green coffee beans produces about 4.4 mg of $CO_2$ and the resulting 96 cal raises the temperature by 0.25 °C (Sivetz and Desrosier, 1979). A high respiration rate, combined with the generation of heat, causes a loss of weight and dry material in the bean, as well as the decomposition of lipids (Rojas, 2009). Factors like temperature, RH, moisture content in the bean and composition of gas in the air affect the stability and quality of the beans. When green coffee is stored for prolonged periods, its quality decreases significantly, and the typical features are flattening and slackening of the cup quality (Selmar et al., 2008).

The so-called “off-notes” are mainly caused by undesired changes within the lipid fractions due to oxidation processes (Kurzrock et al., 2005; Speer and Kölling-Speer, 2006).

Physiology of intermediate seeds during drying and storage is still largely unknown. Significant progress in the understanding of the intermediate storage behaviour has been made by Sacande and coworkers (Sacande et al., 2000, 2001). Desiccation and storage under two RHs (32 and 75 %) were compared using neem seed (*Azadirachta indica*) as a model. It has been shown that the rapid loss of seed viability at 75 % RH in comparison with 32 % RH is associated with an increase in the FFAs content and decreases in the PL content, in the total glutathione content and in the percentage of GSH (Sacande et al., 2000).

Only few studies on coffee seed physiology during storage are available. Selmar et al. (2008) clarified the relationship between the viability of green coffee during storage under standard conditions and the composition of coffee beans, reporting data for quantitative and qualitative changes of free amino acids and soluble sugars. They also found that green coffee, stored with the parchment, remains viable longer than hulled beans and, apparently, longer viability of the seeds correlates to positive effect on preservation of coffee quality. Gradual decreases in content of various sugars (glucose and fructose) and some amino acids are not correlated with the gradual loss of viability. Therefore, the gradual flattening of the aroma during prolonged storage may be correlated with loss of viability and not with changes in the composition of sugars and amino acids. The loss of viability has been correlated to the decline of quality during prolonged storage under standard conditions (Selmar et al., 2008).

Dussert and coworkers (2006) investigated whether changes in antioxidants, sugars and lipids could be detected in coffee seeds dried under various RHs. They demonstrated that oxidative stress, lipid hydrolysis and PL loss were involved in ageing of coffee seeds stored at 20 °C and at intermediate RH (81 %). The origin of oxidative stress in dehydrating organisms, like coffee during processing and storage, is likely due to an uncontrolled formation of ROS as a result of the impairment of the mitochondrial electron transport chain (Oliver et al., 2001), which probably occurs within an interval of intermediary hydration levels where the downregulation of metabolism becomes unco-ordinated (Leprince et al., 1999, 2000). They also investigated the release of FFAs, well-established membrane destabilizing agents (Crowe et al., 1989; Zuidam et al., 1995), which were found to accumulate in ageing coffee seed, suggesting a role for lipase in TAGs hydrolysis (Dussert et al., 2006). These findings are paralleled by a considerable reduction in GSH levels, suggesting that lipid de-esterification might result from free radical attack (Vanbilsen and Hoekstra, 1993). Lipid-ROS interaction has dramatic implications on the internal organization of the cell (Ratajczak and Pukacka,

2005) and on the overall membrane permeability (McDonald, 2000). These processes, directly driven by the end-products of lipid peroxidation, eventually lead to seed death (Wilson and Mcdonald, 1986; McDonald, 1999).

# 7. Aim of the work

As previously described, the colonization of some tree species by phytoplasma implies mechanisms of defence where ROS play a central role. Such activated oxygen species can trigger, directly or indirectly, the disappearance of the symptoms of the disease and, sometimes, also the disappearance of the pathogen from the plant. Thus, the response of plants to pathogen invasion, observed for the recovery phenomenon, is mediated by the oxidative stress.

Coffee beans represent seeds characterized by a high lipid content, are non-dormant and defined “intermediate”. During the storage of these seeds, their metabolism could be active and, in particular, lipase activity can lead to fatty acid release. These compounds can trigger degenerative processes which, in the case of commercial coffee, produce undesired flavours in the final beverage, lowering the cup quality. During coffee seed germination, the coordinated activation of lipolytic enzymes- although not well characterized- occurs, starting from lipid body degradation, involving then the glioxysomes and finally the mitochondria. In these reactions, the lipolytic activity plays a crucial role in providing energy compounds, essential for the heterotrophic metabolism of germinating seed. In this context, unbalanced metabolism could lead to undesired reactions, inducing the oxidation of the lipid fraction and the rise of radical species. The consequent oxidative stress can affect seeds viability and also their storage.

In the light of these considerations, the aims of the work were to study the oxidative stress in plants, following two research topics: in the first one, the response to phytoplasma infection in apple trees was investigated, considering this phenomenon as a model of plant response to biotic stress. In the second part, the lipolytic activities in coffee seeds during either storage and germination was studied to investigate the involvement of such enzymes in the release of free fatty acids and in the induction of degradation processes, as a model of plant abiotic stress.

The studies have been developed through the following lines of research:

1. The involvement of plasma membrane Prxs and oxylipin pathway in the recovery from phytoplasma disease in apple (*Malus domestica*);
2. The characterization of lipolytic activities in green coffee (*Coffea arabica* L.) beans during storage;
3. The lipase activity and antioxidant capacity in green coffee (*Coffea arabica* L.) during germination.

# 8. Results

## *8.1 Attività di perossidasi di superficie e via delle ossilipine in piante di melo soggette a recovery da fitoplasmosi (Petria 2012, 22 (1), 24-31).*

## *Surface peroxidase activity and oxylipin pathway in apple trees recovered from phytoplasma disease*


***Abstract***

Apple trees (*Malus domestica* Borkh) may be affected by apple proliferation (AP), caused by '*Candidatus* Phytoplasma mali'. Some plants can spontaneously recover from the disease, which implies the disappearance of symptoms through a phenomenon known as recovery. Recovered apple trees show the disappearance of the symptoms and pathogens from the canopy, but not from the infected roots, where phytoplasmas persist viable and infectious. Recovery of apple plants is linked to an overproduction of hydrogen peroxide in the phloem tissues. The high levels of hydrogen peroxide in the canopy of recovered apple plants could have a direct antimicrobial effect on pathogens and/or a signalling function, in order to activate defence responses triggered by the phytormones jasmonate and salicylate. In general, it can be stated that salicylate promotes resistance against biotrophic pathogens, whereas the jasmonate pathway induces resistance against necrotrophic pathogens and herbivorous insects. Apple plants, infected by AP-disease, initially show an induced mechanism of defence mediated by salicylate but, subsequently, during the recovery, an increase of jasmonate, sinthesized by the oxylipin pathway, occurs.


### 1. *Il fenomeno del recovery nelle piante*

Il *recovery* è un fenomeno di remissione spontanea dei sintomi in piante affette da fitoplasmosi che è stato descritto in vite, melo e albicocco (Musetti, 2007), sebbene non implichi necessariamente la scomparsa del patogeno dalla pianta ospite (Carraro *et al.*, 2004; R. Osler, comunicazione personale).

Il recovery in piante di melo (*Malus domestica* Borkh) provoca la guarigione dalla proliferazione degli scopazzi (*apple proliferation, AP*), una patologia causata da *Candidatus Phytoplasma mali*. I tipici sintomi della malattia, oltre agli scopazzi, sono rappresentati dalla mancanza di vigore della pianta, dalla presenza di foglie più piccole della norma e con stipole ingigantite, dalla formazione di rosette fogliari e, infine, da una fioritura anomala. Gli effetti

della malattia si ripercuotono, inoltre, sulle dimensioni, sul colore e sul sapore del frutto, rendendolo talvolta non commerciabile. I meli in stato di *recovery* sono caratterizzati dalla scomparsa del patogeno dalla chioma della pianta, mentre a livello radicale il fitoplasma è ancora presente ed infettivo. La probabilità che queste piante siano soggette a nuove infezioni è peraltro quattro volte inferiore rispetto ai meli mai infettati da fitoplasmi, suggerendo il possibile sviluppo di una forma di resistenza indotta (Osler *et al*., 1999).

Recentemente è stato osservato che in melo (Musetti *et al*., 2004), albicocco (Musetti *et al*., 2005) e vite (Musetti *et al*., 2007) il *recovery* da malattie causate da fitoplasmi è associato a una sovrapproduzione di perossido di idrogeno ($H_2O_2$) nelle cellule dei tessuti floematici. Ciò implica il coinvolgimento di specie attivate dell'ossigeno e, in particolare, di $H_2O_2$ prodotto durante la risposta allo stress biotico. In vite, parallelamente ad un elevato livello di $H_2O_2$ in cellule del floema, è stata osservata una diminuzione dell'attività antiossidante legata all'inibizione di catalasi e ascorbato perossidasi (Musetti *et al*., 2007), due dei principali enzimi coinvolti nella detossificazione di $H_2O_2$ nei vari comparti cellulari. Analogamente, in melo è stato riscontrato un basso livello di glutatione ridotto ed una bassa attività di guaiacolo perossidasi (Musetti *et al*., 2010). Tali enzimi e molecole antiossidanti sono in grado di ridurre i danni provocati dalle specie attivate dell'ossigeno (ROS), o di controllare finemente la trasduzione del segnale dipendente dalle stesse. Pertanto, la riduzione dell'attività di tali enzimi antiossidanti può contribuire, indirettamente, all'accumulo di ROS nei tessuti in stato di *recovery* (Mittler *et al*., 2004). Gli elevati livelli di $H_2O_2$ che così si realizzano, potrebbero esercitare una diretta attività antimicrobica contro il patogeno. Diversamente potrebbero, fungere da segnale in grado di attivare risposte difensive tra cui quelle ormonali che sono coinvolte nella difesa da patogeni, quali la sintesi di salicilato (SA) e jasmonato (JA), nonché la deposizione di callosio e proteine P a livello floematico (Musetti *et al*., 2010). Questi ultimi cambiamenti strutturali possono condurre all'occlusione delle placche cribrose dei tubi infetti, al fine di contenere la colonizzazione dei tessuti dell'ospite da parte del patogeno. Nelle stesse piante di melo, in stato di *recovery*, è stata pure osservata, a livello fogliare, una più elevata concentrazione di calcio citosolico rispetto a foglie provenienti da piante sintomatiche e asintomatiche. L'elevato livello di calcio nel floema e l'ambiente ossidante generato dall'accumulo di $H_2O_2$, sembrano così essere due fattori in grado di indurre la deposizione di callosio e proteine P (Musetti *et al*., 2010).

## 2. *Le vie del salicilato e del jasmonato nella risposta delle piante a stress*

La risposta difensiva delle piante ai patogeni comporta anche la sintesi di fitormoni, quali SA e JA. In linea generale, il SA promuove la resistenza verso patogeni biotrofi, mentre il JA è coinvolto nella resistenza contro patogeni necrotrofi e insetti erbivori (Bari and Jones, 2009). Tuttavia gli ormoni vegetali non agiscono singolarmente, ma piuttosto come componenti di una complessa rete di regolazione. Infatti, sebbene le vie biosintetiche di SA e JA siano mutualmente antagoniste, sono stati riportati anche casi di interazione sinergica tra i due fitormoni (Beckers and Spoel, 2006). Si suppone che la loro interazione possa essere dose-dipendente, dando luogo a differenti tipologie di resistenza indotta (Mur *et al.*, 2006). Le risposte difensive causate dall'azione combinata di JA e SA possono essere correlate ad un aumento di produzione di ROS, componenti centrali di un ciclo che si autoalimenta (Torres, 2010).

Il SA può essere sintetizzato attraverso la via dei fenilpropanoidi, in cui l'enzima fenilalanina ammonia liasi (PAL) catalizza il primo passaggio della via biosintetica che comporta la conversione della fenilalanina ad acido trans-cinnamico. Nel passaggio successivo l'acido trans-cinnamico è trasformato in acido benzoico, diretto precursore del SA. Recentemente è stato osservato che le piante, così come i batteri, sono in grado di sintetizzare il SA anche a partire dall'acido corismico, utilizzando l'acido isocorismico come intermedio. Dall'analogia con il meccanismo di sintesi del SA nei batteri, si suppone che una isocorismato-piruvato liasi catalizzi la conversione da isocorismato a SA (Shah, 2003). Oltre al SA, la via dei fenilpropanoidi porta alla sintesi di una vasta serie di composti coinvolti nelle risposte difensive delle piante ai patogeni, quali fenoli, lignine, flavonoidi e fitoalessine.

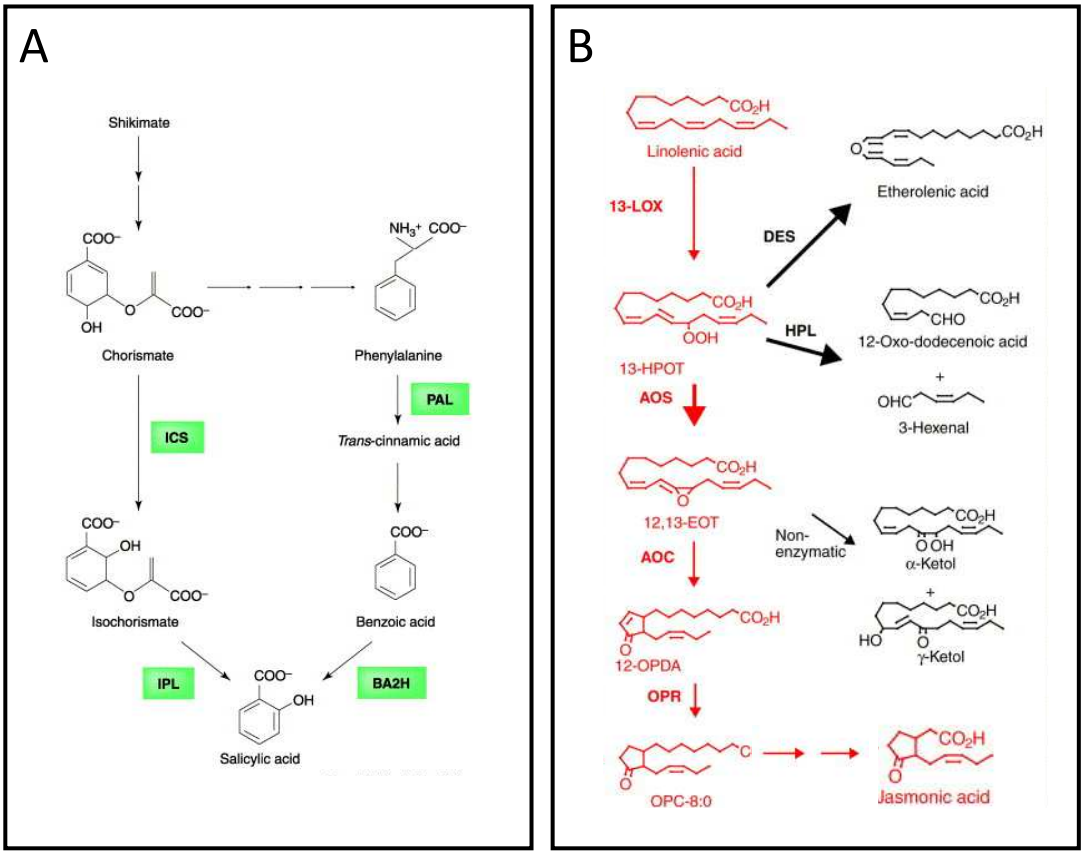


**Fig.1** Vie biosintetiche dei fitormoni salicilato e jasmonato. A, vie di biosintesi del salicilato (*da Shah, 2003*). ICS: isocorismato sintasi; IPL: isocorismato piruvato liasi; PAL: fenilalanina ammonio liasi; BA2H: acido benzoico 2-idrossilasi. B, rappresentazione della via delle ossilipine (*da Howe and Schilmiller, 2002, modificato*). LOX: lipossigenasi; DES: divinil etere sintasi; HPL: idroperossido liasi; AOS: allene ossido sintasi; AOC: allene ossido ciclasi; OPR: acido osso-fitodienoico riduttasi.

Il JA appartiene alla famiglia delle ossilipine (acidi grassi polinsaturi ossigenati), la maggior parte delle quali viene sintetizzata dall'enzima lipossigenasi (LOX), che catalizza l'inserimento di una molecola di ossigeno in posizione 9 o 13 sulla catena idrocarburica dell'acido linolenico o linoleico, portando alla formazione di un acido grasso (9 o 13)-idroperossido. Analisi biochimiche dettagliate hanno evidenziato che gli idroperossidi prodotti dalla LOX sono ulteriormente metabolizzati per sintetizzare una serie di ossilipine in seguito all'attività catalitica di diversi enzimi comprendenti l'allene ossido sintasi, l'idroperossido liasi (HPL), la divinile etere sintasi, la epossi alcol sintasi, la perossigenasi o epossigenasi, la idroperossido riduttasi e la LOX stessa. Le ossilipine sono generalmente sintetizzate *ex-novo* in risposta a ferite meccaniche, causate da erbivori o patogeni, oppure indotte da altri fattori ambientali e di sviluppo (Howe and Schilmiller, 2002). Le idroperossido liasi sono principalmente associate ai cloroplasti ed appartengono alla famiglia

delle proteine CYP74, monossigenasi che catalizzano la scissione degli idroperossidi generando aldeidi volatili e ossoacidi. In base alla specificità per il substrato, le HPL sono suddivise in due sottofamiglie che includono le 13-idroperossidoliasi e le 9/13-idroperossidoliasi. Le 13-HPL rappresentano la sottofamiglia più diffusa nel regno vegetale, dove sembrano svolgere un importante ruolo difensivo contro patogeni ed insetti (Howe and Schilmiller, 2002), oltre ad essere i principali responsabili dell'aroma di molti frutti e delle foglie verdi (Kalua *et al*., 2007).

### 3. *NAD(P)H ossidasi/perossidasi nella risposta ad attacchi di patogeni*

L'induzione delle risposte difensive, mediate da SA e JA, nonché le loro interazioni possono dipendere da segnali trasmessi dalle ROS. Tali molecole reattive possono essere prodotte in vari comparti all'interno della cellula e anche all'interfaccia plasmalemma/parete cellulare. Nello specifico, l'enzima NAD(P)H ossidasi, i cui omologhi in piante sono definiti *respiratory burst oxidase homologs* (Rboh), è stato proposto come il responsabile del *burst* ossidativo a livello apoplastico nella maggior parte delle interazioni pianta-patogeno (Torres and Dangl, 2005). Rboh catalizza la produzione di superossido attraverso il trasferimento di un elettrone all'ossigeno molecolare (reazione univalente) usando l'NAD(P)H come donatore di elettroni (Choi *et al*., 2007). Le NAD(P)H ossidasi nei vegetali sono caratterizzate da un motivo strutturale *EF-hand* all'N-terminale, un sito di legame per il calcio che probabilmente regola l'attivazione dell'enzima (Bolwell *et al*., 2002). L'enzima contiene a livello citosolico un motivo di legame per il FAD e per l'NAD(P)H; inoltre presenta sei eliche di transmembrana che coordinano due gruppi eme. Questi ultimi sono necessari per trasferire gli elettroni attraverso la membrana all'accettore extracellulare, l'ossigeno, e sono legati alla terza e alla quinta elica transmembrana attraverso residui di istidina (Glyan'ko and Ischenko, 2010). Recentemente è stato osservato che i membri della famiglia Rboh, durante le risposte difensive, mediano la produzione di ROS a livello apoplastico, dove sono, inoltre, coinvolti nello sviluppo e nella risposta a stress ambientali (Torres and Dangl, 2005).

In aggiunta alle NAD(P)H ossidasi vegetali, le perossidasi sono state proposte come fonte alternativa di ROS (Apel and Hirt, 2004; Choi *et al*., 2007). Molte perossidasi sono localizzate a livello extracellulare e sono classificate come libere, legate ionicamente, oppure legate covalentemente alla parete cellulare. Le perossidasi possono agire in due modi distinti: in presenza di $H_2O_2$ e di substrati fenolici sono coinvolte nella sintesi di lignina e di altri polimeri fenolici; se il substrato fenolico è sostituito da NAD(P)H o composti correlati, si verifica una reazione a catena che produce $H_2O_2$ mediante l'ossidazione dell'NAD(P)H

(Vianello and Macrì, 1991; Apel and Hirt, 2004). Numerosi studi hanno dimostrato la presenza di attività NAD(P)H perossidasica anche in vescicole di membrane plasmatiche. La funzione di queste perossidasi appare essere simile a quella delle perossidasi di parete coinvolte nella risposta difensiva. Le perossidasi appartengono ad una superfamiglia di enzimi ubiquitari in funghi, piante e vertebrati, contengono una ferri-protoporfirina IX come gruppo prostetico e ossidano numerosi substrati (sostanze fenoliche, ascorbato, ammine, acido indolacetico, ecc.) in presenza di $H_2O_2$ (Vianello *et al*., 1997; Mika and Luthje, 2003). Perossidasi solubili, sia intracellulari sia extracellulari, sono state caratterizzate in dettaglio, mentre ancora poche conoscenze sono disponibili relativamente alle perossidasi di plasmalemma. Tuttavia, attività perossidasiche a livello di membrana plasmatica sono state più volte evidenziate in piante superiori (Vianello *et al*., 1997; Mika and Luthje, 2003).

## 4. *Il coinvolgimento della via del jasmonato nel recovery in piante di melo*

Nonostante le evidenze sperimentali sopra descritte, le basi fisiologiche del *recovery* sono ancora poco conosciute. Pertanto, è stato condotto uno studio al fine di descrivere i *pattern* biochimici associati a questo fenomeno.

Sulla base di recenti risultati, siamo in grado di proporre un meccanismo bifasico di resistenza acquisita per spiegare il fenomeno del *recovery* in piante di melo. Nella prima fase, in cui le piante sono infette e manifestano sintomi evidenti, i principali segnali sembrano associati all'attivazione di perossidasi di superficie (generazione di $H_2O_2$) e alla via dei fenilpropanoidi (produzione di SA); nella seconda fase, coincidente con il *recovery*, le perossidasi di superficie restano ancora attive, la risposta della pianta sembra dipendere dalla via delle ossilipine che porta alla sintesi di JA, mentre si riduce contestualmente la biosintesi di SA. La caratteristica comune tra le due fasi è, quindi, rappresentata dall'attività di perossidasi di superficie.

La sovrapproduzione di $H_2O_2$ è un fenomeno ampiamente riconosciuto quale primo segnale di risposta in diverse piante infette da funghi, batteri, virus e viroidi (Hiraga *et al*., 2001). Tuttavia, l'ossidazione del NAD(P)H in piante *recovered* non risulta completamente inibita dal KCN, un noto inibitore delle proteine eme, mentre l'attività delle perossidasi di superficie è stimolata proprio a seguito della scomparsa dei sintomi, cioè in fase di *recovery*. Pertanto, si suppone che durante il *recovery* le perossidasi di superficie giochino un ruolo preponderante rispetto alle NAD(P)H ossidasi, flavoproteine insensibili al KCN le quali, durante il *recovery*, sembrano aver già esaurito il loro ruolo nell'induzione dell'iniziale *burst* ossidativo. Proponiamo, dunque, che un aumento di attività perossidasica, congiuntamente

alla riduzione della capacità di detossificare le ROS (Musetti *et al*., 2004), siano responsabili della sovrapproduzione di $H_2O_2$ rilevata durante il *recovery*.

Nel nostro modello le piante sintomatiche mostrano un aumento di SA nei tessuti fogliari, suggerendo che questo ormone possa fungere da marker della fase iniziale di sviluppo della malattia. Il coinvolgimento del SA in risposta al patogeno trova conferma nell'aumento di attività della PAL, un enzima chiave nella biosintesi del SA, che è stato osservato in piante di melo infette. D'altro canto, utilizzando approcci biochimici in piante infette da fitoplasmi, è già stata riportata un'alterazione (espressa come attività della PAL) nel contenuto di polifenoli, nonché una variazione di altri metaboliti secondari (Musetti *et al.*, 2010).

Come abbiamo visto, nelle piante *recovered* la risposta mediata dal SA è rimpiazzata da quella dipendente dal JA, il cui livello risulta più elevato in tali piante dove sembra svolgere un ruolo chiave. La via delle ossilipine appare così meno attiva nelle fasi iniziali della malattia, mentre sembra svolgere una funzione preponderante durante la succesiva fase di remissione dei sintomi. L'aumento di JA appare correlato all'incremento di attività della LOX, che risulta stimolata in foglie di melo *recovered*, rispetto a quella di piante malate. Un andamento diverso è stato invece osservato per la HPL, la cui attività è stimolata sia nelle foglie sintomatiche sia in quelle *recovered*. Pertanto, la risposta al patogeno è ancora attiva nelle piante asintomatiche. Ciò potrebbe indicare che, sebbene quest'enzima non sia strettamente coinvolto nel fenomeno del *recovery*, la produzione di composti volatili con funzione inibitoria potrebbe svolgere un ruolo nel contrastare l'invasione della chioma da parte del patogeno. Questo dato appare peraltro in accordo con l'osservazione che il *recovery* da fitoplasmi in melo si accompagna alla scomparsa del patogeno dalla chioma, ma non dalle radici (Musetti *et al.*, 2004).

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## *8.2 Involvement of plasma membrane peroxidases and oxylipin pathway in the recovery from phytoplasma disease in apple* (Malus domestica).

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**Abstract**

Apple trees (*Malus domestica* Borkh) may be affected by apple proliferation (AP), caused by '*Candidatus* Phytoplasma mali'. Some plants can spontaneously recover from the disease, which implies the disappearance of symptoms through a phenomenon known as recovery. In this paper it is shown that NAD(P)H peroxidases of leaf plasma membrane-enriched fractions exhibited a higher activity in samples from both AP-diseased and recovered plants. In addition, an increase in endogenous SA was characteristic of the symptomatic plants, since its content increased in samples obtained from diseased apple trees. In agreement, phenylalanine ammonia lyase (PAL) activity, a key enzyme of the phenylpropanoid pathway, was increased too. Jasmonic acid (JA) increased only during recovery, in a phase subsequent to the pathological state, and in concomitance to a decline of salicylic acid (SA). Oxylipin pathway, responsible for JA synthesis, was not induced during the development of AP-disease, but it appeared to be stimulated when the recovery occurred. Accordingly, lipoxygenase (LOX) activity, detected in plasma membrane-enriched fractions, showed an increase in apple leaves obtained from recovered plants. This enhancement was paralleled by an increase of hydroperoxide lyase (HPL) activity, detected in leaf microsomes, albeit the latter enzyme was activated in either the disease or recovery conditions. Hence, a reciprocal antagonism between SA- and JA-pathways could be suggested as an effective mechanism by which apple plants react to phytoplasma invasions, thereby providing a suitable defence response leading to the establishment of the recovery phenomenon.

**Introduction**

Plants may express an inducible systemic defence when infected by pathogens. Systemic acquired resistance (SAR) is an example of an inducible defence mechanism that is activated in distal organs of plants in response to local pathogen infections (Durrant and Dong, 2004). SAR is triggered by a pathogen that causes tissue necrosis, either as part of a hypersensitive response or as symptom of disease (Conrath, 2006). It involves the signal

molecule salicylic acid (SA) and the accumulation of pathogenesis-related proteins (PR proteins) (Durrant and Dong, 2004). Another type of inducible defence mechanism is the induced systemic resistance (ISR). The latter is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, in which local defences establish a state of heightened resistance throughout the plant against subsequent attack (van Loon et al. 1998). Interestingly, ISR does not involve the accumulation of PR proteins or SA signalling (Pieterse et al. 2009), but instead relies on a pathway regulated by jasmonic acid (JA) and ethylene (ET). In particular, JA is the main product of an important biosynthetic pathway initiated by 13-lipoxygenases. These enzymes (LOXs) add molecular oxygen to polyunsaturated fatty acids to yield the corresponding fatty acid hydroperoxides that are substrates for other enzymes, such as hydroperoxide lyase, allene oxide synthase, divinyl ether synthase, etc. (Mosblech et al. 2009), to produce an array of molecules known as oxylipins. Among them, JA is a plant hormone involved in resistance to necrotrophic pathogens, insect attack, wounding and in establishing systemic resistance (Andreou and Feussner, 2009; Feussner and Wasternack, 2002; Howe and Jander, 2008; Lopez et al. 2011). The involvement of phytohormones (such as SA and JA together with ET and abscisic acid), as primary signals in the regulation of plant defence, is well established (Pieterse et al. 2009; Verhage et al. 2010). Generally speaking, SA promotes resistance against biotrophic pathogens, whereas the JA/ET pathway induces resistance against necrotrophic pathogens and herbivorous insects (Bari and Jones, 2009). Despite this simplified model, the different plant hormones do not act alone, but rather as components of a complex and interconnected regulatory network. In addition, although SA and JA/ET defence pathways are mutually antagonistic, evidence of synergistic interactions has also been reported (Beckers and Spoel, 2006; Pieterse et al. 2009; Moore et al. 2011). Recent acquisitions suggest even a complex network between hormones in an interplay that could be dose-dependent, leading to a different type of inducible resistance (Mur et al. 2006). In addition, defence responses modulated by JA and SA depend on the pathogen lifestyle (Seilaniantz et al. 2011). Both SA- and JA-induced defence responses could be mediated by an increase of reactive oxygen species (ROS), a central component of a self-amplifying loop (Torres, 2010).

Some plants (e.g. grapevine, apple, pear and apricot), affected by phytoplasmas (plant-pathogenic prokaryotes), could evolve into a spontaneous remission of symptoms, known as recovery, which may or may not involve the disappearance of the pathogen from the host (Carraro et al. 2004; R. Osler, personal communication). This phenomenon can occur in apple trees (*Malus domestica* Borkh) affected by apple proliferation (AP), which is caused by

"*Candidatus* Phytoplasma mali" ("*Ca.* P. mali") (Seemüller and Schneider, 2004). The typical symptoms of the disease in the plants are lack of vigour, witches' broom, presence of smaller leaves with enlarged stipules, leaf-rosette formations and abnormal flowers. Furthermore, the effect of the pathogen attack is deleterious on size, colour and taste of the apples, which sometimes are not suitable for commercialization. The symptoms occurrence in infected plants can be irregular year after year; witches' brooms are typical in recently infected plants that often recover in the following years (Seemüller et al. 2011). Recovered apple trees show the disappearance of the symptoms and pathogens from the canopy, but not from the infected roots, where phytoplasmas persist viable and infectious (Carraro et al. 2004). In the recovered plants the probability of a subsequent canopy re-infection is about four times lower than that of plants which have not been previously infected, suggesting the occurrence of an induced resistance mechanism (Osler et al. 2000). Variation in symptoms intensity is correlated to the seasonal fluctuation of phytoplasma presence and concentration in the canopy of infected apple trees and probably related also to the loss of sieve tubes functionality that occurs during winter in the aerial part. On the contrary, phytoplasmas can survive in the roots of infected plants were the phloem functionality is not interrupted during winter. In fact, detectability of phytoplasmas by DAPI and immunofluorescence in the aerial part of infected apple trees decreases during winter, but remains high in the roots (Seemüller et al. 1984; Loi et al. 2002).

Recovery of apple plants is linked to an overproduction of hydrogen peroxide, which appears to be localised in the phloem tissue (Musetti et al. 2004). This implies the involvement of ROS during the process and, in particular, a role for $H_2O_2$ during this biotic stress. The high levels of $H_2O_2$ in the canopy of recovered apple plants could have a direct antimicrobial effect on pathogens and/or a signalling function, by activating defence genes (Torres, 2010), leading to the deposition of callose and phloem-proteins (Musetti et al. 2010).

Despite the above-mentioned evidence, the physiological basis of the recovery is still poorly understood. Therefore, the biochemical pattern associated to this phenomenon was studied in more detail. This aim was accomplished by examining some enzymatic activities related to oxidative stress (NAD(P)H oxidase/peroxidase) and to the synthesis of two stress hormones, such as SA (phenylalanine ammonia lyase, PAL) and JA (lipoxygenase, LOX and hydroperoxide lyase, HPL), in different sub-cellular fractions (microsomes, plasma membrane-enriched fractions, or soluble fractions), obtained from healthy, diseased and recovered apple leaves. Finally, the levels of SA and JA in leaf extracts were also measured.

## Materials and methods

### Source of material and phytoplasma detection

The experimental apple orchard (*Malus domestica* Borkh, cv. Florina grafted on M26 rootstock), grown under organic conditions, was located in Lavariano (Friuli Venezia Giulia region, North Eastern Italy) and established in 1988. First symptoms of AP were recorded in 1990 and an epidemic peak of the disease was observed in the '90s with 76 % of plants showing symptoms for at least one year. First cases of recovery were observed in 1992 (Carraro et al. 2004). During the period 1988-2010 all the test plants were checked three times during the year for symptoms expression (in July, September and December) and some of them were randomly analyzed every year for "*Ca*. P. mali" presence in the leaves by serological or molecular methods (Loi et al. 2002; Lorenz et al. 1995). In addition, all the sampled plants were tested for the presence of "*Ca*. P. mali" in the roots, allowing to discriminate three groups of plants: symptomatic (for at least two consecutive years), healthy from the beginning and recovered (previous symptomatic plants that have not shown symptoms at least for two years). At the beginning of September, samples of roots and leaves, collected from the 15 selected individuals, belonging to the three groups of plants, were analyzed by nested-PCR in order to confirm the phytosanitary status of the plants. For "*Ca*. P. mali" detection, nucleic acids were extracted from roots and leaf midribs following the Doyle and Doyle (1990) modified procedure and a nested-PCR assay, based on ribosomal protein primers rpAP15f2/rp(I)R1A and rpAP15f/rpAP15r, was applied (Martini et al. 2007). Amplicons were submitted to restriction fragment length polymorphism (RFLP) analyses using AluI and DraI endonucleases and agarose gel electrophoresis was adopted to visualize RFLP patterns. After "*Ca*. P. mali" detection on individual plants, for physiological studies, a bulk of mature leaves from symptomatic, recovered and healthy plants were randomly collected and stored at -80 °C. The enzymatic activities, except those for membrane marker detection and chlorophyll content, were analyzed in healthy, diseased and recovered apple leaves.

### Isolation of microsomes and plasma membrane-enriched fractions

Microsomes were isolated as previously described by Zhang et al. (2006), with minor changes. Leaves (25 g) were ground in liquid nitrogen and homogenised with 200 ml of extraction buffer containing 100 mM Tris-HCl (pH 7.5), 250 mM sucrose, 2 mM EDTA, 1 mM $MgCl_2$, 10 % (v/v) glycerol, 0.3 % (w/v) bovine serum albumine, 5 mM ascorbic acid, 1

mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 1,4-dithioerythritol (DTE) and 0.2 % (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through 50 µm nylon mesh and centrifuged at 12 000 *g* (Sorvall RC-5B centrifuge, SS-34 rotor, DuPont Company, DE, USA) for 20 min. The supernatant was filtered through cotton gauze and centrifuged at 100 000 *g* (Beckman LE80K ultracentrifuge, Ty 70ti rotor, Beckman Coulter, Fullerton, CA, USA) for 40 min. The pellet (microsomal fraction) was resuspended either in 25 mM Tris-HCl (pH 7.5), 250 mM sucrose and 1 mM DTE, for the enzymatic activity assays, or in 5 mM $K_2HPO_4$ /$KH_2PO_4$ (pH 7.8), 250 mM sucrose and 4 mM KCl (buffer A, final volume ca. 3 ml) for the further purification of plasma membranes by the aqueous polymer two-phase partitioning (Larsson et al. 1987). The system for plasma membrane purification was constituted by 6.5 % (w/w) polyethylene glycol and 6.5 % (w/w) dextran T-500 in buffer A. Three successive cycles of partitioning yielded the final upper phase that was diluted in 200 ml washing buffer containing 25 mM Tris-HCl (pH 7.5), 250 mM sucrose and centrifuged at 120 000 *g* (Ty 70ti Beckman rotor, Beckman Coulter, Fullerton, CA, USA) for 70 min. The pellet (plasma membrane-enriched fractions, PMEF) was resuspended in 500 µl washing buffer. All the above steps were carried out at 4 °C.

**Membrane marker enzyme activities and chlorophyll content**

Vanadate-sensitive (plasma membrane marker enzyme) and bafilomycin $A_1$-sensitive (tonoplast marker enzyme) ATPase activities were assayed as previously described (Macri et al. 1994). Antimycin A-insensitive cytochrome *c* reductase (marker enzyme for endoplasmic reticulum) and cytochrome *c* oxidase (marker enzyme for mitochondria) activities were detected as described by (Hodges and Leonard, 1974; Lord, 1983). Chlorophyll content was evaluated as chloroplast marker according to the method of Arnon (1949).

**NAD(P)H oxidase/peroxidase activity**

NAD(P)H oxidase/peroxidase activity was evaluated as NAD(P)H oxidation, determined spectrophotometrically (Agilent 8453 spectrophotometer, Agilent, Santa Clara, CA, USA) as decrease of absorbance at 340 nm. The incubation buffer consisted of 40 mM Na-acetate (pH 5.5), 250 mM sucrose and 50 µg of PMEF protein in a final volume of 2 ml, in the presence of 2 mM $MnCl_2$ and 100 µM salicylhydroxamic acid (SHAM). The reactions were started by the addition of 100 µM NAD(P)H and proceeded at 25 °C. Inhibition of NAD(P)H oxidase/peroxidase activity was achieved by the addition of either 200 U $ml^{-1}$ superoxide dismutase (SOD) or 1 mM KCN.

**Lipoxygenase activity**

LOX activity was determined spectrophotometrically (Agilent 8453 spectrophotometer, Agilent, Santa Clara, CA, USA) as increase of absorbance at 234 nm, following the generation of hydroperoxyoctadecanoic acids (HPODE). LOX activity was assayed at different pH values, using the appropriate buffer as follow: 0.1 M Na-acetate (pH 5.0-5.5); 0.1 M MES-KOH (pH 6.0-7.0); 0.1 M EPPS-KOH (pH 7.5-8.5); 0.1 M borate-KOH (pH 9.0). The reaction mixture consisted of 25 µg of PMEF protein in a final volume of 2 ml incubation buffer. Linoleic acid was dissolved under nitrogen flux in 5 mM borate buffer (pH 9.0), 0.1 mM EDTA, and 9.6 % ethanol (v/v), 0.1 mM DTE; a few drops of Tween 20 were added to solubilise fatty acid. The solution was then divided into small aliquots (250 µl), which were flushed with nitrogen and stored at -20 °C. The reaction was started by the addition of 250 µM linoleic acid and proceeded at 25 °C. An extinction coefficient ($\varepsilon_M$) of 25 000 $M^{-1}$ $cm^{-1}$ was used to estimate the amount of conjugated dienes. Production of HPODE was evaluated at different concentrations of linoleic acid, ranging from 25 to 250 µM.

**Regioselectivity of HPODE in PMEF**

The products of PMEF-linked LOXs were additionally analysed to clearly identify and quantify 13- and 9-HPODE by RP-HPLC. Isomers were produced following the addition of linoleic acid, in the presence or absence of nordihydroguaiaretic acid (NDGA).

Experimental mixtures were buffered at the pH exhibiting the maximal LOX activity. The reaction was started by the addition of 250 µM linoleic acid to 500 µg of PMEF protein dissolved in 5 ml of the incubation buffer, in the presence or absence of 100 µM NDGA. The solution was stirred for 5 min at 25 °C. The reaction mixture was acidified to pH 2 with 1 N HCl and then extracted twice with 10 ml of hexane and isopropanol (95:5 v/v). The solvent used for the extraction contained 0.22 mM butylhydroxytoluene (BHT), as an internal standard, because it exhibits high absorbance at the wavelength of detection (234 nm) and does not interfere chromatographically with the analytes. Moreover, it also has antioxidant properties and shares hydrophobicity and extraction features with HPODE. The extract was dried with sodium sulphate, evaporated under a nitrogen flow and resuspended in 200 µl of the HPLC mobile phase. Products were subjected to RP-HPLC (Agilent 1200 series instrument), following the protocol described by Patui et al. (2007) with minor modifications. HPLC was performed on a Zorbax Eclipse XDBC18 column (5 mm, 4.6 mm x 150 mm, Agilent). The mobile phase consisted of solvent A (water with 0.25 % v/v acetic acid) and

solvent B (acetonitrile). The gradient elution procedure was: 0–70 min, 50 % of B; 70–72 min, a linear gradient from 50 to 80 % of B; 72–90 min, 80 % of B; 90 to 92 min from 80 to 50 % of B. The flow rate was 0.5 ml/min. Peak areas were measured at 234 nm. The isomers were identified by comparison with 9- and 13-HPODE and quantified by using BHT as an internal standard.

**Hydroperoxide lyase activity**

HPL activity was determined spectrophotometrically at 25 °C, using 13-HPODE as substrate, by monitoring the decrease in absorbance at 234 nm (Agilent 8453 spectrophotometer, Agilent, Santa Clara, CA, USA). Enzyme assays were performed over a wide pH interval, ranging from 5.0 to 9.0, using the incubation buffers above described. The reaction mixture consisted of 20 µg of microsomal membrane protein, in a final volume of 2 ml. The reactions were started by the addition of 13-HPODE at concentrations ranging from 0.1 µM to 0.6 µM.

**Phenylalanine ammonia-lyase activity**

PAL activity was measured according to the method of Chen et al. (2009), with minor modifications. Leaves (1 g) were homogenised with mortar and pestle in 5 ml of extraction buffer (50 mM Tris–HCl buffer, pH 8.8, 15 mM β-mercaptoethanol, 5 mM EDTA, 5 mM ascorbic acid, 10 µM leupeptin, 1 mM PMSF and 0.15%, w/v, PVPP). The homogenate was centrifuged (SS-34 Sorvall rotor, Newtown, CT, USA) at 20 000 *g* for 20 min at 4 °C to remove cell debris. One ml of the supernatant was filtered through a 5 ml column of Sephadex G-25 to remove compounds interfering with the optical assay and to change the buffer to 0.1 M borate (pH 8.2). The protein containing fractions (1.5 ml) of the eluate were used for the enzymatic activity determination. The reaction mixture (1 ml) contained 50 mM Tris-HCl buffer (pH 8.8), 3.6 mM NaCl, 100 µg of protein and was started by the addition of 16 mM *L*-phenylalanine. Incubation was performed at 37 °C for 1 h and the reaction stopped by the addition of 150 µl of 6 M HCl. The reaction mixture was then centrifuged for 5 min at 12 000 *g* to pellet denatured proteins, and its absorbance was measured at 290 nm. The activity of PAL was expressed as nmol of cinnamic acid $mg^{-1}$ prot $h^{-1}$.

**Salicylic acid determination**

Leaf samples (0.5 g) were ground in liquid nitrogen and incubated at 95 °C in a stirring solution containing 5 mM triethanolamine (pH 7.5), 20 µM EDTA and 0.4 µM $CaCl_2$.

Toluic acid (0.4 mM) was added as the internal standard in a final volume of 5 ml. The above solution was incubated for 1 h and then centrifuged at 13 000 *g* (SS-34 Sorvall rotor, Newtown, CT, USA) for 12 min. Supernatant was collected and acidified with 0.05 volumes of 4 mM HCl solution, then warmed up at 95 °C for 1 h and cooled at room temperature. Total SA was extracted with 2 volumes of ethyl acetate/hexane/isopropanol (50:50:1, v/v/v). The organic phase, containing free SA, was dried under nitrogen and dissolved in 200 µl mobile phase composed by water/acetic acid/methanol (64:1:35, v/v/v). SA was measured by RP-HPLC on a Zorbax Eclipse extra dense bonding (XDB)-C18 column (5 µm, 4.6 mm x 150 mm, Agilent 1200 series instrument, Santa Clara, CA, USA), equipped with a binary pump delivery system, coupled to a DAD, using an isocratic elution with water/acetic acid/methanol (64:1:35, v/v/v) at a flow rate of 1 ml $min^{-1}$. SA was measured at 280 nm. The compound was identified by comparison with a certified standard solution (VWR International, Lutterworth, UK). The quantification of SA was referred to a calibration curve obtained by a standard of toluic acid, using the internal standard procedure.

**Jasmonic acid determination**

Leaf samples (0.2 g) were ground in liquid nitrogen and homogenised in 2 ml 80 % (v/v) aqueous methanol, 0.2 % (v/v) acetic acid at 4 °C for 30 min. For quantification of JA, 10 µg of methyl JA was added as the internal standard. Samples were centrifuged at 13 000 *g* for 10 min. The supernatants were then collected and analysed by RP-HPLC on a Zorbax Eclipse XDB-C18 column (5 µm, 4.6 mm x 150 mm, Agilent 1200 series instrument, Santa Clara, CA, USA). The separation was carried out according to Kramell et al. (2000) with the following solvent system: A = methanol, B = water with 0.2 % acetic acid. A gradient elution was performed at a flow rate of 1 ml $min^{-1}$: 50 % of A for 7 minutes, followed by an increase up to 90 % of A for 15 minutes, an isocratic run at 90% of solvent A for 2 min and, finally, reconditioning up to 50 % of A for 5 min. The absorbance for JA was monitored at 202 nm.

**Protein determination**

The protein content was determined by the Bradford method (Bradford, 1976), according to manufacturer's instructions and using crystalline bovine serum albumin as a standard.

## Results

### Apple tree source and phytoplasma detection

The selected AP-symptomatic plants showed typical symptoms of the disease: witches' brooms, small leaves with enlarged stipules and crop reduction, whereas recovered and never symptomatic plants were undistinguishable. Samples collected from roots of symptomatic and recovered apple trees (Fig. 1, panel A) and leaf samples collected from symptomatic plants (Fig. 1, panel B) resulted to be positive in nested-PCR assays. RFLP analysis on the obtained amplicons confirmed the presence of "*Ca*. P. mali" subtype rpX-A (results not shown). Roots from healthy plants and leaves from both recovered and healthy apple trees did not show any PCR products.

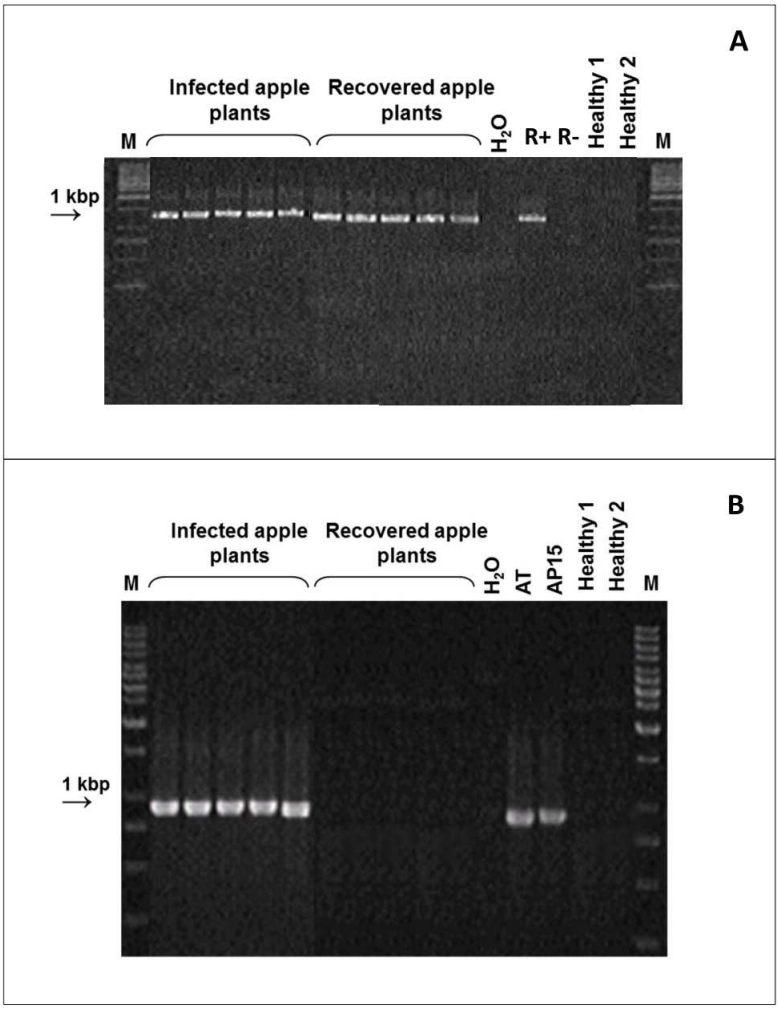


**Figure 1.** Electrophoretic fractionation on agarose gel of nested-PCR amplified products, obtained using rpAP15f2/rp(I)R1A and rpAP15f/rpAP15r primer pairs on DNA extracted from infected and recovered apple plants (individually tested), and healthy 1 and healthy 2 (a bulk of five never symptomatic plants tested). Panel A: samples from roots; panel B: samples from leaf midribs. R+, positive control, roots from AP infected apple maintained in greenhouse; R-, negative control, roots from healthy apple maintained in greenhouse; AT and AP15, reference strains from periwinkle isolates; M, DNA ladder (Fermentas, Lithuania).

**Characterization of membrane fractions**

Microsomes were isolated from apple leaves and were also further processed to obtain PMEF, whose level of purification was characterised by the analysis of enzymatic and molecular markers (Table 1). ATPase activity was not inhibited by bafilomycin $A_1$ in both microsomes and PMEF, while vanadate was more effective in PMEF, reaching approx. 70 % of inhibition. The activity of antimycin A-insensitive cytochrome *c* reductase (endoplasmic reticulum marker enzyme) was found to be about 20 % in PMEF, with respect to microsomes. Furthermore, when compared to PMEF the microsomal fraction showed a higher activity of cytochrome *c* oxidase (a mitochondrial marker enzyme). Chlorophyll content (detected as marker of chloroplasts) was significantly lower in PMEF with respect to microsomes.

Therefore, these results show that PMEF were enriched in plasma membrane, almost devoid of tonoplast, slightly contaminated by endoplasmic reticulum and, although to a lesser extent, by mitochondrial and chloroplast membranes.

| | | MF | | PMEF | |
|---|---|---|---|---|---|
| Marker | Additions | | % | | % |
| ATPase | | 23.1 | 100 | 41.3 | 100 |
| | 500 μM vanadate | 14.2 | 62 | 12.4 | 30 |
| | 0.2 μM bafilomycin $A_1$ | 27.5 | 119 | 39.6 | 96 |
| Antimycin A-insensitive cytochrome *c* reductase | | 33.3 | | 7.4 | |
| Cytochrome *c* oxidase | | 55.8 | | 29.8 | |
| Chlorophyll | | 4.4 | | 1.5 | |

**Table 1.** Marker enzyme activities in microsomes (MF) and plasma membrane-enriched fractions (PMEF) isolated from apple leaves. Enzymatic activity values are expressed as nmol $mg^{-1}$ prot $min^{-1}$; data of ATPase activity are also expressed as percentage in the absence (set at 100 %) or in the presence of inhibitors; chlorophyll content is expressed as mg $ml^{-1}$. Data are means of at least three replicates.

**NAD(P)H oxidase/peroxidase activity related to oxidative stress**

Since NAD(P)H oxidase/peroxidase are enzymes typically localised at the level of cell wall/plasma membrane interface, their activity was detected in PMEF. This activity was not detectable at neutral pH in any samples (results not shown), but at pH 5.5, $Mn^{2+}$ and SHAM induced the oxidation of both NADH and NADPH at comparable levels (Fig. 2). This activity exhibited an increasing rate for NAD(P)H oxidation of 202 (trace a), 309 (trace b) and 565 (trace c) nmol $mg^{-1}$ protein $min^{-1}$ in PMEF samples from healthy, diseased and recovered plants, respectively. NAD(P)H oxidation was strongly inhibited by the addition of either superoxide dismutase (SOD) or potassium cyanide (KCN). The addition of KCN (typical hemoprotein inhibitor) caused a decrease in NAD(P)H oxidation, which resulted to be inhibited of 65, 77 and 59% in PMEF samples from healthy, diseased and recovered plants, respectively. The same PMEF samples, in the presence of SOD, exhibited a stronger inhibition of 80, 84 and 71 %, respectively.

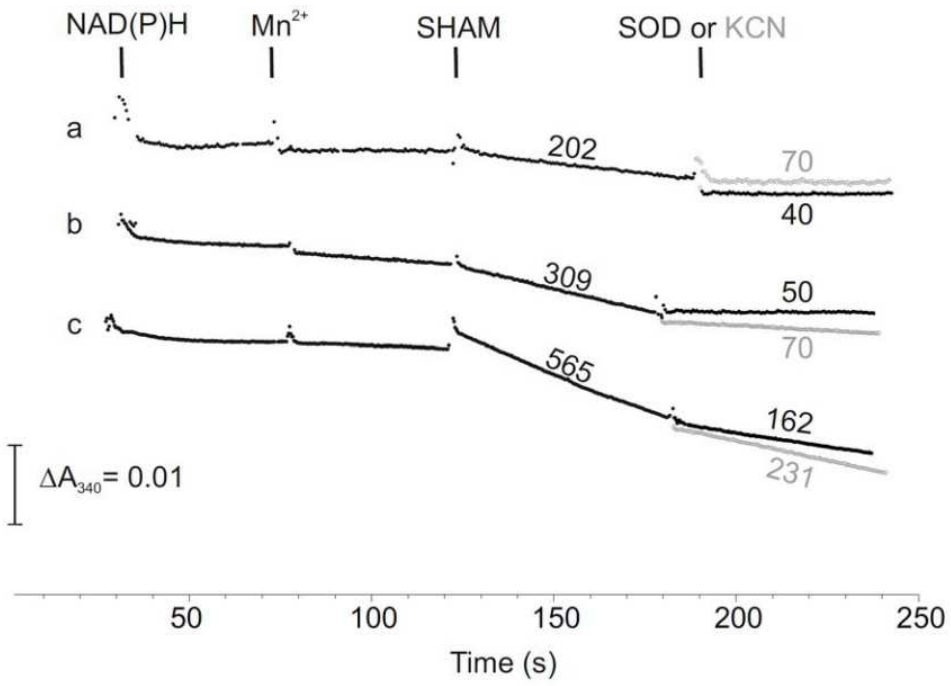


**Figure 2**. NAD(P)H oxidase/peroxidase activity, monitored as decrease of absorbance at 340 nm, in PMEF obtained from leaves of healthy (a), diseased (b) and recovered (c) apple plants. The activity was evaluated at pH 5.5, after addition of 2 mM $MnCl_2$ and 100 µM SHAM. Other additions were either 200 U ml-1 SOD or 1 mM KCN. Values are expressed as nmol NAD(P)H oxidised $mg^{-1}$ protein $min^{-1}$. Data are means of at least three replicates.

**Lipoxygenase and hydroperoxide lyase activities as key enzymes of the oxylipin pathway**

LOXs catalyse the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids possessing a (1*Z*,4*Z*)-pentadiene sequence, yielding the corresponding fatty acid hydroperoxides. In plants, α-linolenic (18:3) and linoleic (18:2) acids are the most common substrates for LOXs. Several LOX isoforms may occur in different cell compartments (e.g. cytosol, stroma and vacuole) or associated to membranes (e.g. plasma membrane and chloroplast) (Mosblech et al. 2009). LOX activity was measured in PMEF to avoid the interfering activity of HPL, which is mainly associated with chloroplasts. In addition, LOX activity associated to plasma membrane appears to be more promptly involved in the response of the plant to pathogen (Blée 2002). Fatty acid hydroperoxides can be further degraded by HPL, leading to the production of aldehydes and $\omega$-oxo acids. Since HPL is a membrane-bound enzyme (present mainly in chloroplasts) (Mosblech et al. 2009), its activity was analysed in microsomes, which contained also a fraction of such membranes. LOX and HPL activities were characterised and compared, as a function of pH. LOX showed an activity in a pH range between 5.0 and 7.0, exhibiting two peaks, at pH 5.0 and 6.5, in PMEF from healthy leaves (Fig. 3, panel A). This pattern was also found in PMEF from recovered plants (optimum at pH 6.5), whereas those from diseased plants showed only a peak of activity at pH 5.5, suggesting a differential expression of LOX isoform(s) during pathogenesis and recovery, being the recovered plants able to regain a LOX array similar to healthy ones. In microsomal fractions HPL was detected in a broad range from pH 6.0 to 9.0, showing a peak at pH 7.5 for all samples (Fig. 3, panel B). HPL activity was higher in microsomes from diseased and recovered plants than in healthy ones.

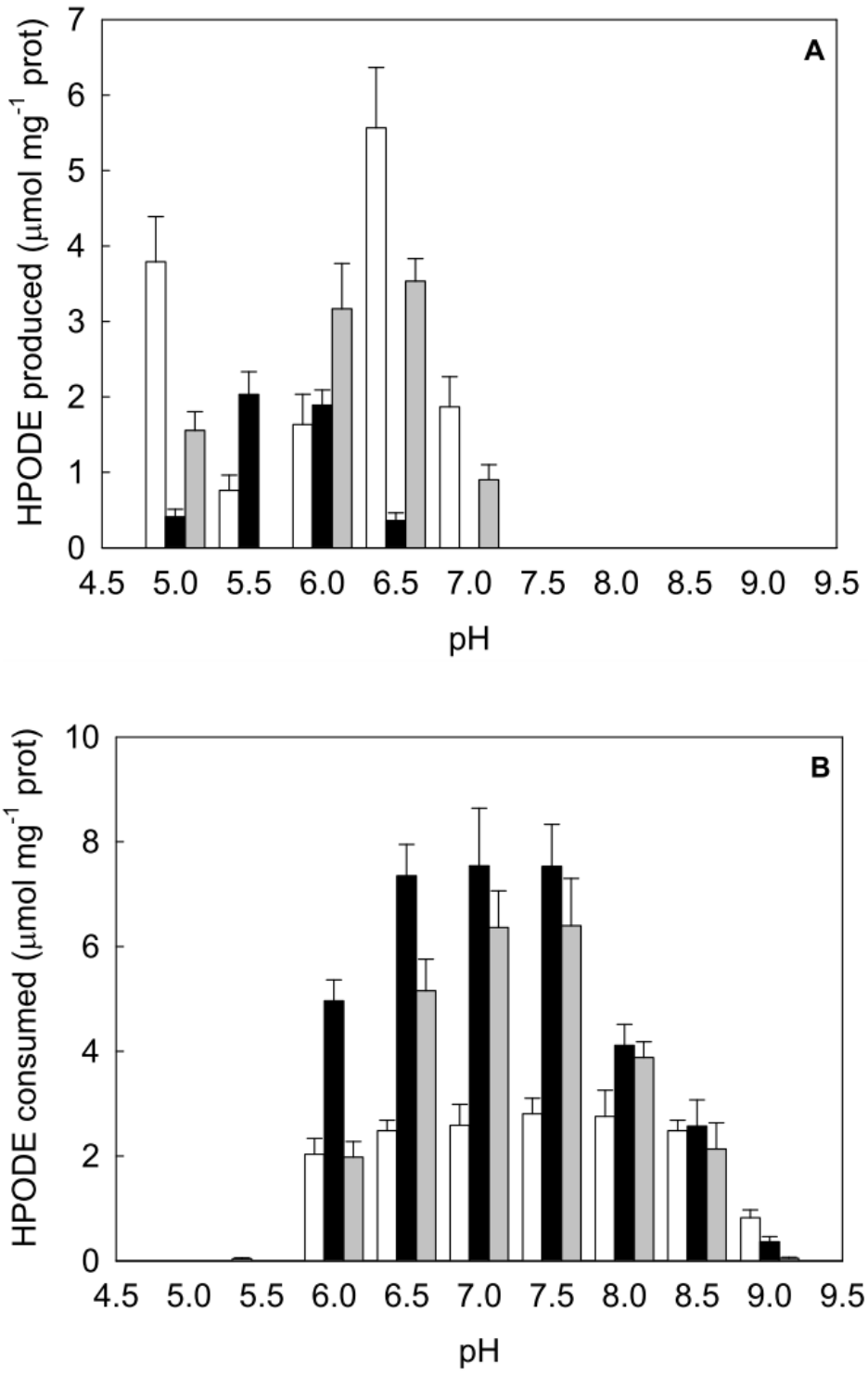


**Figure 3**. Effect of pH on LOX and HPL activity in healthy (white columns), diseased (black columns) and recovered (grey columns) apple leaf samples. Panel A, linoleic acid-dependent LOX activity in PMEF. Amounts of HPODE produced are given in µmol $mg^{-1}$ protein. Panel B, HPODE-dependent HPL activity in microsomes. Amounts of HPODE consumed are given in µmol $mg^{-1}$ protein. Values represent the mean ± SD.

LOX activities were evaluated at their pH optima, as linoleic acid-dependent conjugated diene formation in PMEF samples from healthy, diseased and recovered plants (Fig. 4, panel A). LOX activity resulted to be higher in PMEF from recovered than healthy or diseased apple leaves. This activity was further evaluated at increasing concentrations of linoleic acid, ranging from 25 to 250 µM, at optimum pHs (Fig. 4, panel B). The initial rate values could fit the Michaelis-Menten equation, whose kinetic parameters, $V_{max}$ and $K_M$, are summarized in Table 2. The maximal velocities were quite similar in healthy and recovered PMEF, whereas this value decreased of about 70 % in samples from diseased plants. Recovered plants exhibited the lowest $K_M$ values, indicating the highest catalytic efficiency (Table 2).

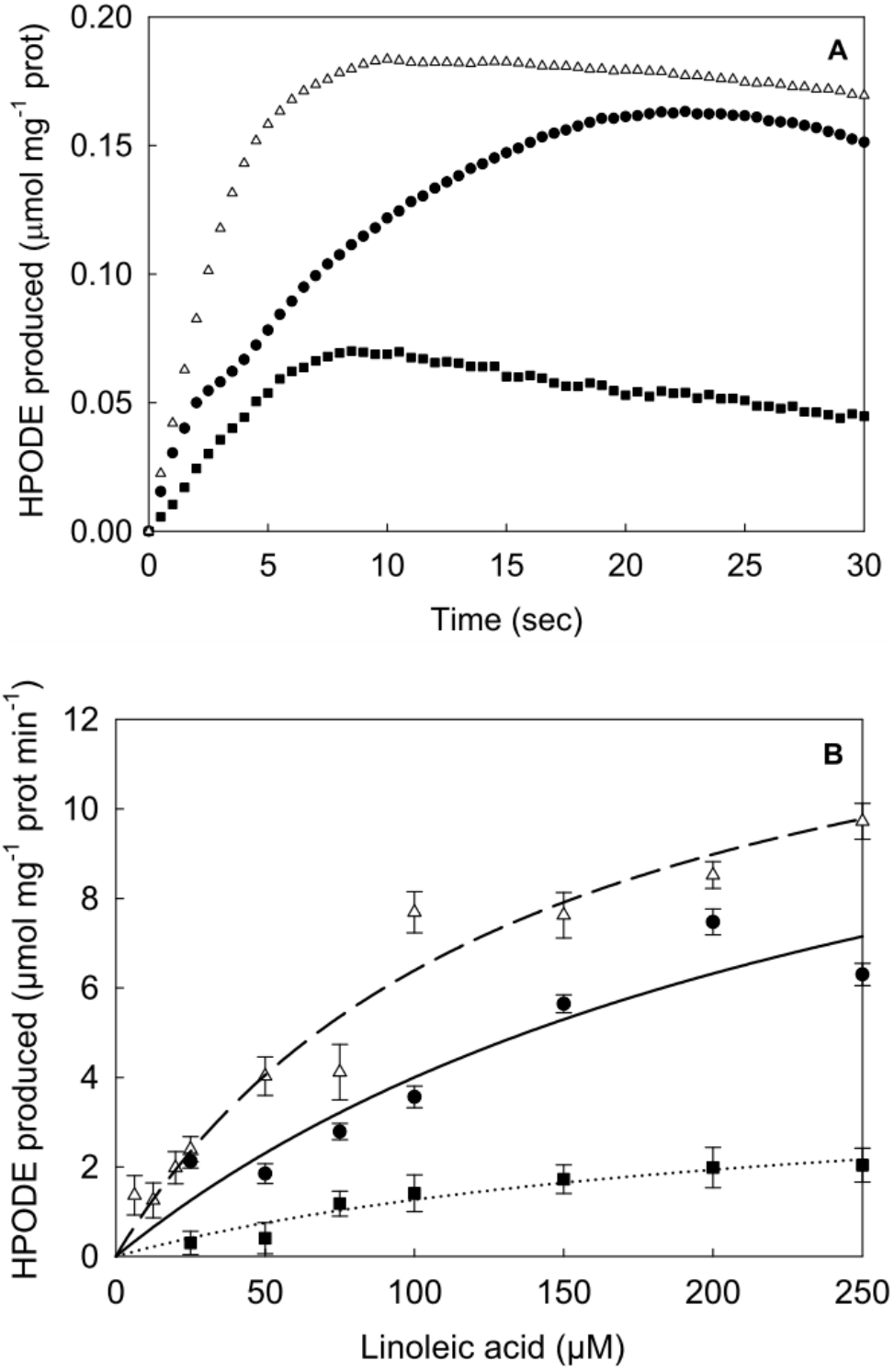


**Figure 4.** LOX activity in PMEF obtained from healthy (closed circles), diseased (closed squares) and recovered (open triangles) apple leaves. Panel A, LOX activity was evaluated as hydroperoxide (conjugated diene) formation, at optimum pHs. Panel B, LOX activity as a function of linoleic acid concentration. Values of HPODE produced are given in µmol $mg^{-1}$ protein $min^{-1}$. Data represent the mean ± SD.

| | LOX activity | | HPL activity | |
|---|---|---|---|---|
| | $V_{max}$ µmol $mg^{-1}$ prot $min^{-1}$ | $K_M$ [µM] | $V_{max}$ µmol $mg^{-1}$ prot $min^{-1}$ | $K_M$ [µM] |
| Healthy | 13.7 | 238 | 7.3 | 1.71 |
| Diseased | 4.1 | 226 | 4.3 | 0.16 |
| Recovered | 15.1 | 137 | 3.7 | 0.14 |

**Table 2**. Kinetic parameters ($V_{max}$ and $K_M$) of lipoxygenase (LOX) and hydroperoxide lyase (HPL) activities measured in PMEF and microsomal fractions, respectively, obtained from apple leaves.

LOX products were characterized by RP-HPLC analysis (Fig. 5). All the samples showed a mixture of 9- and 13-HPODE. In particular, the amount of HPODE, due to LOX activity, was calculated as the difference between the whole amount of HPODE detected in the assay and that of HPODE detected in the presence of NDGA (NDGA-sensitive HPODE production). The NDGA-sensitive HPODE production (LOX activity) was characterized by a ratio between 9 and 13 isomers of HPODE that was near 4:1 in healthy samples and 1.6:1 in diseased leaves, whereas the ratio was 2.3:1 in recovered samples, implying the presence of different LOX isoforms.

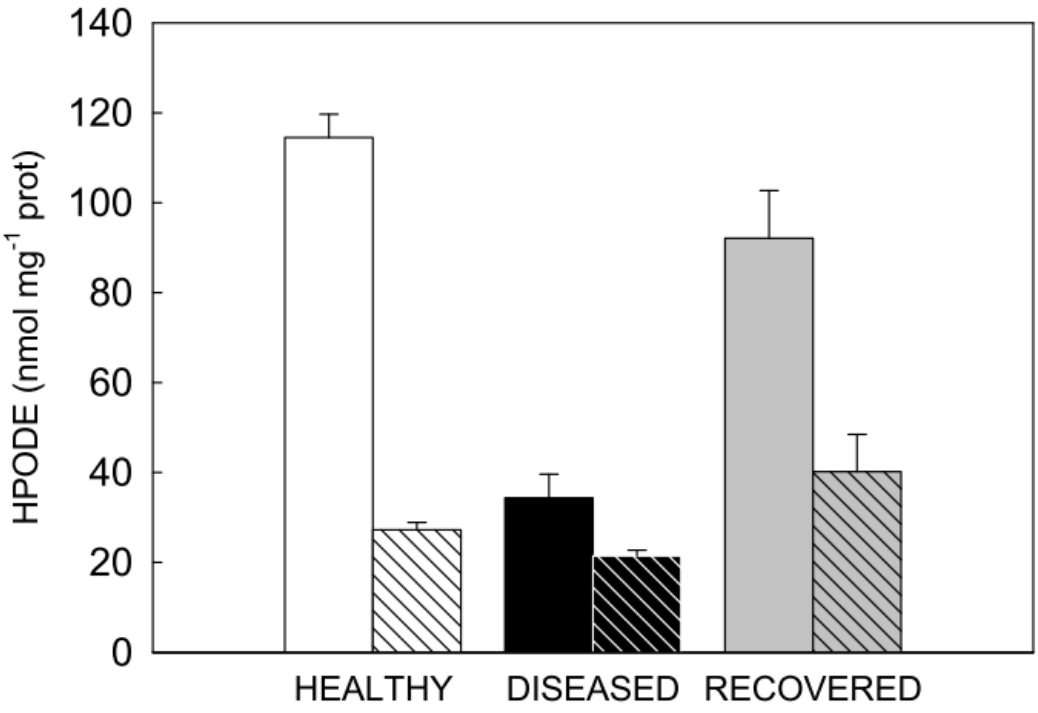


**Figure 5.** HPODE isomer production by PMEF-linked LOXs obtained from healthy (white columns), diseased (black columns) and recovered (grey columns) apple leaves. Different isomers are indicated as full columns (13-HPODE) or dashed columns (9-HPODE). Data represent the mean ± SD.

HPL activity was evaluated at pH 7.5 in microsomes obtained from the three types of leaves (healthy, diseased and recovered), because in all cases it was found to be the optimal value (see Fig. 3, panel B). HPL activity was increased in samples from diseased and recovered plants, when compared to samples from healthy ones. However, no difference could be detected between samples from diseased and recovered plants (Fig. 6, panel A). This pattern was confirmed by examining HPL activity as a function of HPODE concentration (Fig. 6, panel B). As for LOX enzyme, the Michaelis-Menten equation allowed to calculate the $V_{max}$ and $K_M$ values (Table 2). Both kinetic parameters were higher in healthy leaves when compared with diseased and recovered samples which exhibited a very similar pattern.

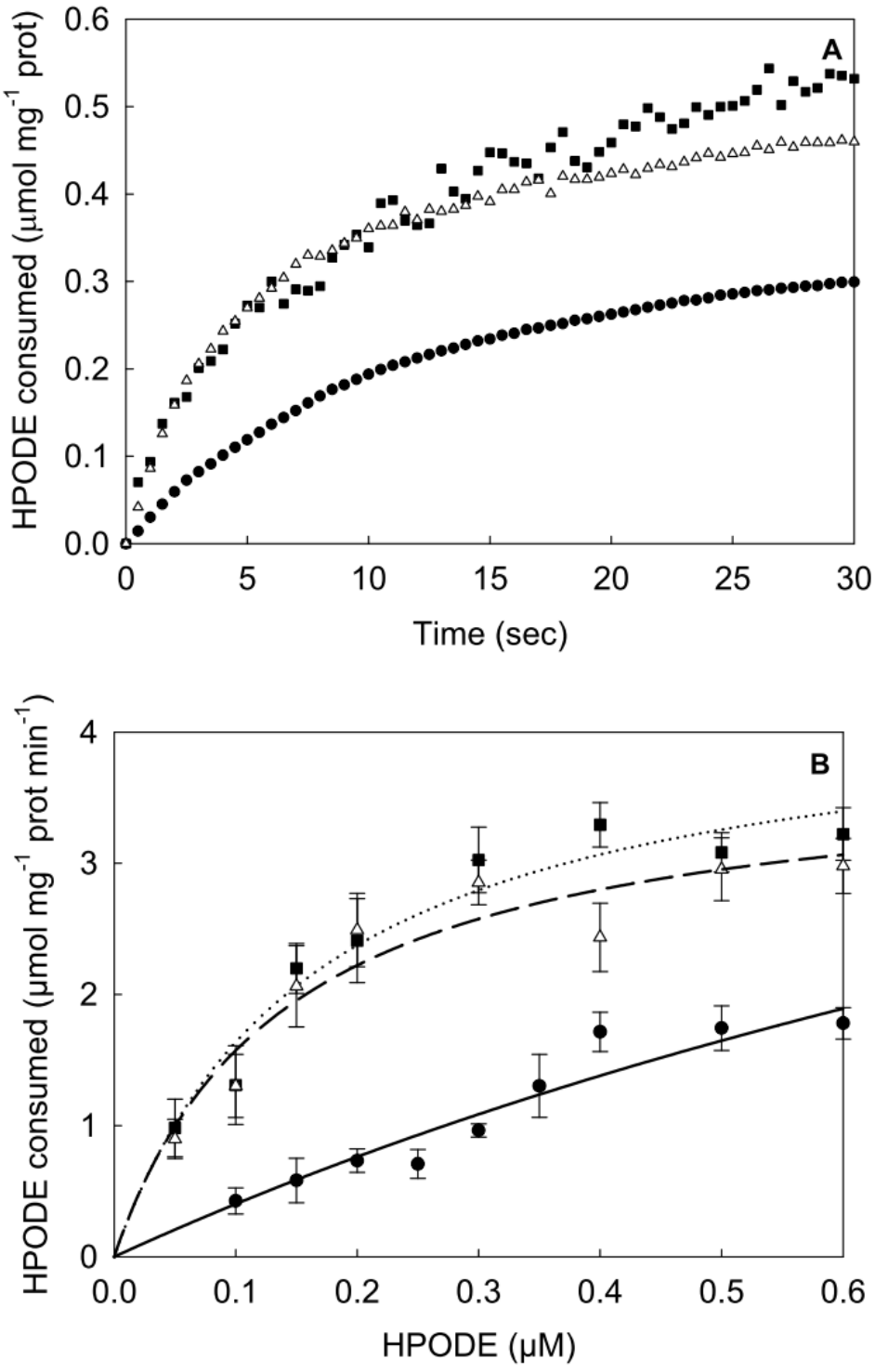


**Figure 6.** HPL activity in microsomal fractions from healthy (closed circles), diseased (closed squares) and recovered (open triangles) apple leaves. Panel A, HPL activity was evaluated as 13 HPODE degradation at pH 7.5. Panel B, HPL activity evaluated as a function of 13-HPODE concentration. Values of HPODE consumed are given in µmol $mg^{-1}$ protein $min^{-1}$. Data represent the mean ± SD

**Phenylalanine ammonia-lyase activity**

PAL catalyses the first step in the biosynthesis of a wide array of phenolic compounds, therefore it has a major influence on phenylpropanoid biosynthesis (Clark et al. 1994). The first metabolite in the phenylpropanoid pathway is *trans*-cinnamic acid, precursor of a wide range of plant defence compounds, such as lignin, flavonoids, phenolics, phytoalexins and, in particular, SA. Since the involvement of SA in defence signalling has been extensively characterised in dicotyledonous plants (Chaturvedi and Shah, 2007), PAL activity was determined in order to verify whether this biosynthetic pathway is involved in recovery response.

PAL activity was detected in soluble fractions obtained from leaves of healthy, diseased and recovered plants (Fig. 7). PAL, evaluated as production of *trans*-cinnamic acid, in healthy samples showed an appreciable activity that was slightly higher in diseased leaves, but was significantly lower in recovered samples.

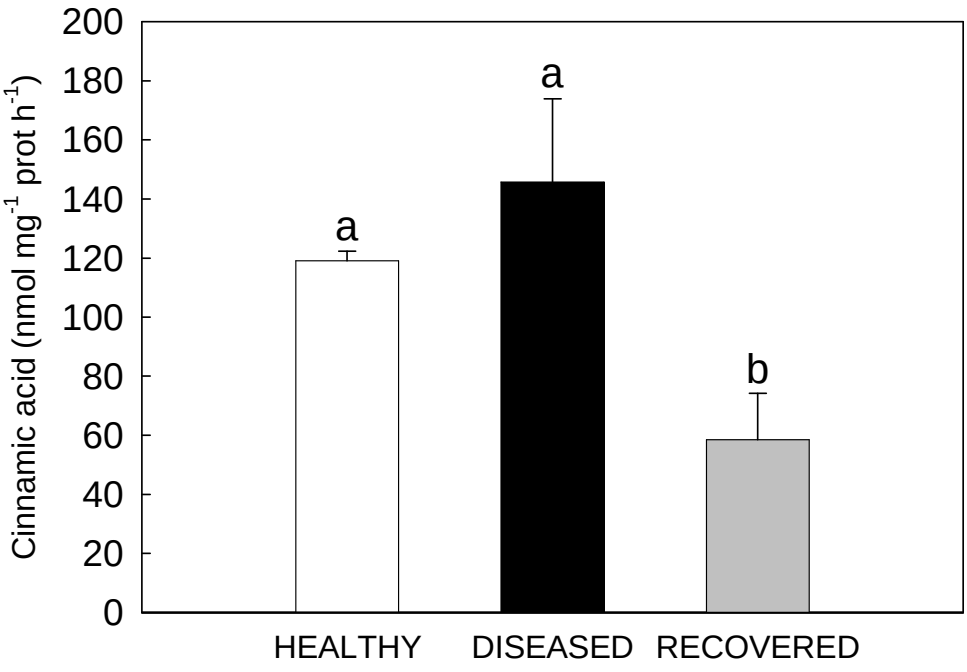


**Figure 7.** PAL activity in the soluble fractions of healthy (white columns), diseased (black columns) and recovered (grey columns) apple leaves. PAL activity was evaluated as cinnamic acid formation. Amount is given in nmol $mg^{-1}$ protein $h^{-1}$. Values represent the mean ± SD.

**Determination of SA and JA**

The phytohormones SA and JA play a key role in regulating the activation of the basal plant resistance to pathogens. The endogenous level of free SA was detected by RP-HPLC, after extraction and acid hydrolysis from leaves of healthy, diseased and recovered apple plants. This method allowed a simultaneous measurement of free and glucose-conjugated SA (Fig. 8, panel A). The SA level was found to be increased in diseased leaves of about 30 %, with respect to recovered leaves, which in turn showed a SA concentration comparable to that of healthy plants. JA was detected by RP-HPLC, after extraction from healthy, diseased and recovered apple leaves (Fig. 8, panel B). Its concentration, in leaf samples obtained from diseased plants, was four times lower, if compared with that of recovered plants, whereas healthy plants exhibited an intermediate concentration of such a hormone. The chromatograms are shown in Appendix S1 as Supporting Information.

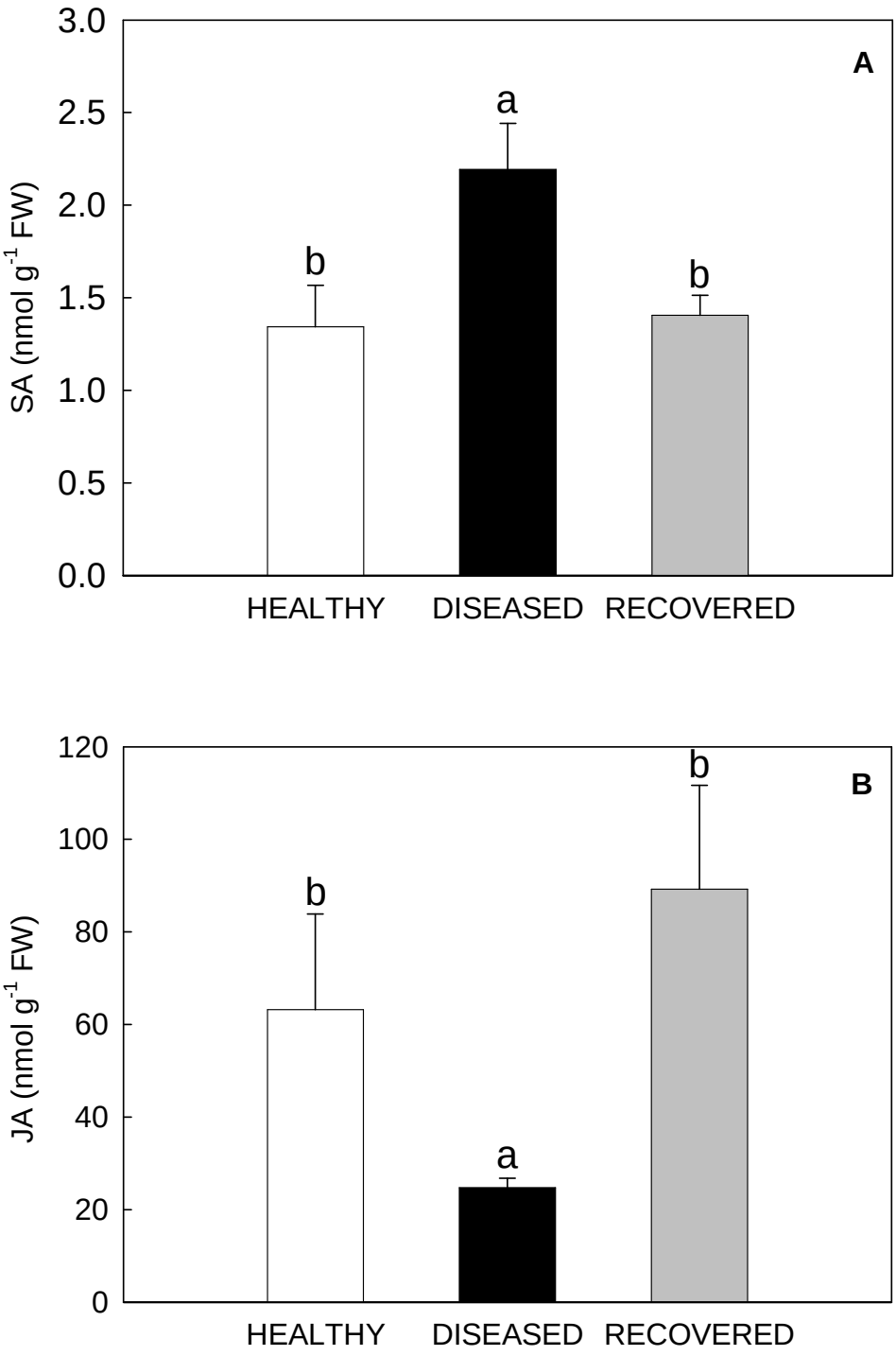


**Figure 8.** Analysis of SA and JA level in healthy (white columns), diseased (black columns) and recovered (grey columns) apple leaves. Both compounds were detected by RP-HPLC and the amounts are given in nmol $g^{-1}$ fresh weight. Panel A, SA content; panel B, JA content. Values are means of three replicates ± SD.

**Discussion**

Recovery consists in a spontaneous (transient or permanent) remission from disease symptoms in plants that were previously symptomatically affected by a given pathogen (Carraro et al. 2004). This phenomenon has been observed in economically important species, such as apple, apricot and grapevine (Carraro et al. 2004; Musetti et al. 2004; Musetti et al. 2010), as well as pear (R. Osler, personal communication). In the case of apple, recovery is accompanied by the disappearance of phytoplasmas from the canopy, but not from the roots (Musetti et al. 2004). Multiple factors, such as low levels of antioxidants (reduced glutathione) and guaiacol peroxidase, accumulation of phloem proteins and callose deposition, are involved in the recovery of apple plants, although the phenomenon is not still completely understood (Musetti et al. 2010). The above-mentioned observations, together with the fact that recovered plants can be re-infected in nature to a lesser extent (Osler et al. 1999), indicate that a type of systemic acquired resistance could be responsible for the recovery.

This study was aimed at clarifying some physiological aspects of the recovery phenomenon observed in the interaction between "*Ca*. P. mali" and host plants. Therefore, the following samples were considered during the development of the disease: healthy, diseased ("*Ca*. P. mali"-infected) and recovered plants. Symptomatic plants exhibited increased levels of plasma membrane NAD(P)H peroxidases, leading to the synthesis of $H_2O_2$, and an increased amount of SA. In the second phase, coincident with the recovery, plasma membrane peroxidases were still active, but the response was shifted towards the oxylipin pathway, leading to JA production, with a concomitant decline of the synthesis of SA. Therefore, plasma membrane peroxidase activity was increased during both the disease manifestation and the recovery phenomenon; such enzymes could lead to the production of $H_2O_2$, a typical signal in several plants infected with fungi, bacteria, viruses and viroids (Hiraga et al. 2001). Plasma membrane peroxidase activity appears to be further stimulated after the disappearance of the symptoms (recovery). Nevertheless, in recovered plants NAD(P)H oxidation was not completely abolished by the addition of KCN, a known hemoprotein inhibitor. Thus, during recovery, plasma membrane peroxidases appear to be induced simultaneously with NAD(P)H oxidases, flavoproteins that are not inhibited by KCN. Several NAD(P)H oxidases are typically characterised by a $Ca^{2+}$-binding motif (Torres et al. 1998), implying a direct activation by $Ca^{2+}$ ions. Interestingly, we observed that NAD(P)H oxidase activity increased in recovered samples, where $Ca^{2+}$ concentration was remarkably higher with respect to healthy and diseased plants as shown by Musetti et al. (2010). Therefore, we suggest that an

increase of peroxidase and oxidase activity, together with a decreased ROS scavenging capacity (Musetti et al. 2004), could be responsible for the $H_2O_2$ overproduction observed during recovery. In this context, NAD(P)H-mediated $H_2O_2$ generation could act directly as an antimicrobial defence or indirectly as a signal molecule, inducing the disappearance of the pathogen from the canopy. However, these NAD(P)H oxidases do not seem to belong to those involved in the "oxidative burst", because this activity was not inhibited by DPI (data not shown). On the other hand, the oxidative burst is a short and transient phenomenon occurring at the beginning of the plant/pathogen interaction (Shah 2005).

In our model, AP-symptomatic trees showed an increase of SA content in leaf tissues, suggesting that this hormone could act as a marker for the onset of AP. Besides the involvement of SA in the response of the plant to pathogens, phytoplasma-infected apple exhibited, in agreement, an increase of PAL activity. In this context, it is noteworthy to underline that PAL, peroxidases and polyphenoloxidases are referred to as "pathogenesis related proteins", since they are constitutively present, but increase during pathogen infections (van Loon et al. 2006). On the other hand, an alteration in polyphenol content, as well as other secondary metabolites (expressed as PAL activity), has already been reported in phytoplasma-infected plants, using biochemical approaches (Musetti et al. 2010) and, indirectly, in grapevine by transcriptional data (Albertazzi et al. 2009; Hren et al. 2009), which allow to infer the involvement of polyphenols in the response against phytoplasmas (Margaria et al. 2010). Similarly, also in virus-infected tobacco, SAR is reduced or enhanced by silencing or over-expressing PAL, respectively (Felton et al. 1999). The results of this work showed that the trend of polyphenols and salicylate were coincident with the development of the host-pathogen interaction, since high polyphenol levels were associated with the symptomatic phase (Choi et al. 2004) and would be related to PAL activity increase. These observations are in agreement with recent findings obtained on *Citrus sinensis* (L.) Osbeck, during the initial phase of "yellow shoot disease", also known as "citrus greening". The causal agent of such a disease is *Ca.* Liberibacter asiaticus, a non-culturable bacterium that similarly to phytoplasma is phloem-limited (Albrecht and Bowman 2008). This pathogen is able to induce a dramatic increase of the Myb-like transcriptional regulator family. The expression of the Myb-like factor, a known inducer of SA-mediated hypersensitive response to bacterial pathogens, appears to be characteristic for the symptomatic stage of the disease, while it seems not to be linked to the subsequent tolerance phase (recovery) (Albrecht and Bowman 2012).

In the case of recovered apple plants, the SA-mediated response was then replaced with that dependent on JA, thus suggesting the interplay between the two hormones. JA level

increased in recovered apple plants, implying the key role of the oxylipin pathway, whose function in defence responses is well-known. It involves signalling and/or synthesis of protective compounds, such as antibacterial and wound-healing agents (Blée 2002; Feussner and Wasternack 2002). Nevertheless, the oxylipin pathway is less active during the development of AP, but it is rather involved during the symptom remission phase, of which JA could be considered a marker. The increase of JA is remarkable as the increase of LOX activity, since this activity was found to be stimulated in PMEF from recovered apple leaves. Interestingly, LOX exhibited at least two pH optima (5.5 and 6.5, for diseased and recovered plants, respectively), suggesting the presence of two isoenzymes (acidic and sub-neutral) acting in different contexts. It appears that the neutral isoform was active during the manifestation of recovery, while diseased plants exhibited just the acidic activity. It is noteworthy that the acidic LOX seems to accomplish the same function observed in other biotic stresses (Kohlmann et al. 1999; Veronico et al. 2006), where it is often classified as LOX2. These observations are confirmed by the isoenzyme characterization by RP-HPLC. The acidic LOX showed a ratio 1.6:1 of its products (13- and 9-HPODE). Conversely, the HPODE produced by the sub-neutral LOXs shifted the ratio towards 13-HPODEs. Furthermore, a higher amount of 9-hydroperoxides in "*Ca*. P. mali"-infected samples is in agreement with previous results, showing that 9-HPODEs are clearly involved in defence and pathogen attacks (Gao et al. 2007). Since lipid hydroperoxides, produced by enzymatic and non-enzymatic activities, are highly reactive, they have to be rapidly converted by HPL and allene oxide synthase into several metabolites, which may be precursors for JA, methyl jasmonate, conjugated dienoic acids and volatile aldehydes (Mosblech et al. 2009).

Values of $K_M$ parameters for LOX were two orders of magnitude higher than those of HPL activity for all three types of plants, indicating a lower catalytic efficiency for LOX with respect to HPL enzyme. The latter feature suggests that the LOX-catalyzed step is the limiting factor in the oxylipin pathway activation induced by "*Ca*. P. mali" infection.

Our results show that HPL activity increased in both diseased and recovered samples, indicating that its activation was ubiquitous during the host-pathogen interaction. In addition, this persisting activity observed in recovered plants resembles the peroxidase-oxidase activity previously described, demonstrating that the pathogen response was still present in plants that have got over the disease. For this reason, although this enzyme does not seem specifically correlated to the recovery phenomenon, it might indicate that toxic HPL-derived products could play a role in counteracting further pathogen invasions in the canopy by altering volatile emission involved in insect vectors attraction (Plotto et al. 2010; Sugio et al. 2011). This is in

agreement with the observation that the recovery from phytoplasmas in apple plants is correlated with the disappearance of the pathogen from the canopy, but not from the roots (Musetti et al. 2004). Therefore, the compounds produced by HPL activity could limit the spread of the pathogen, or decrease the colonisation by insect vectors, acting as repellents (Eigenbrode et al. 2002).

These results are consistent with previous works showing that the infection by "*Ca.* L. asiaticus" in *C. sinensis* (L.) Osbeck induced a differential gene expression during disease development. In particular, JA- and ethylene-dependent systemic resistance was down-regulated during early compatible host-pathogen interaction, while only few defence related genes were up-regulated leading to an ineffective defence response. Further on (13-17 weeks), a more substantial increase in gene expression of bacterial-induced peroxidase and LOX were observed (Albrecht and Bowman 2008). In addition, different genes, such as VvLox (encoding LOX in *Vitis vinifera*), were found to be differentially expressed in both healthy and infected by Bois noir ('BN') phytoplasma grapevine (Hren et al. 2009). The involvement of LOX in disease response is also supported by studies on "yellow shoot disease" induced by "*Ca.* L. asiaticus" affecting sweet orange, where up-regulation of LOX expression was detected by proteomic analysis (Fan et al. 2011). Finally, in Madagascar periwinkle, infected by phytoplasmas, the JAZ protein (transcriptional repressor of jasmonate-responsive genes), resulted down-regulated, suggesting again the involvement of this hormone in the response to phytoplasma infection (De Luca et al. 2011). Further proof may also be inferred by recovery induction in 'BN' and Flavescence doreè-infected grapevines after partial uprooting and pulling, agronomical practices that induce stress in plants (Romanazzi and Murolo 2008). This type of mechanical stress is very similar to wounding, which leads, as known, to the activation of the oxylipin pathway.

In conclusion, apple plants, infected by "*Ca.* P. mali", initially show a mechanism of defence associated to a high SA content, but during the subsequent recovery an increase of JA synthesized by the oxylipin pathway occurs. The activation of plasma membrane peroxidases and the production of $H_2O_2$ act as essential components in the signal transduction cascade leading to defence reactions.

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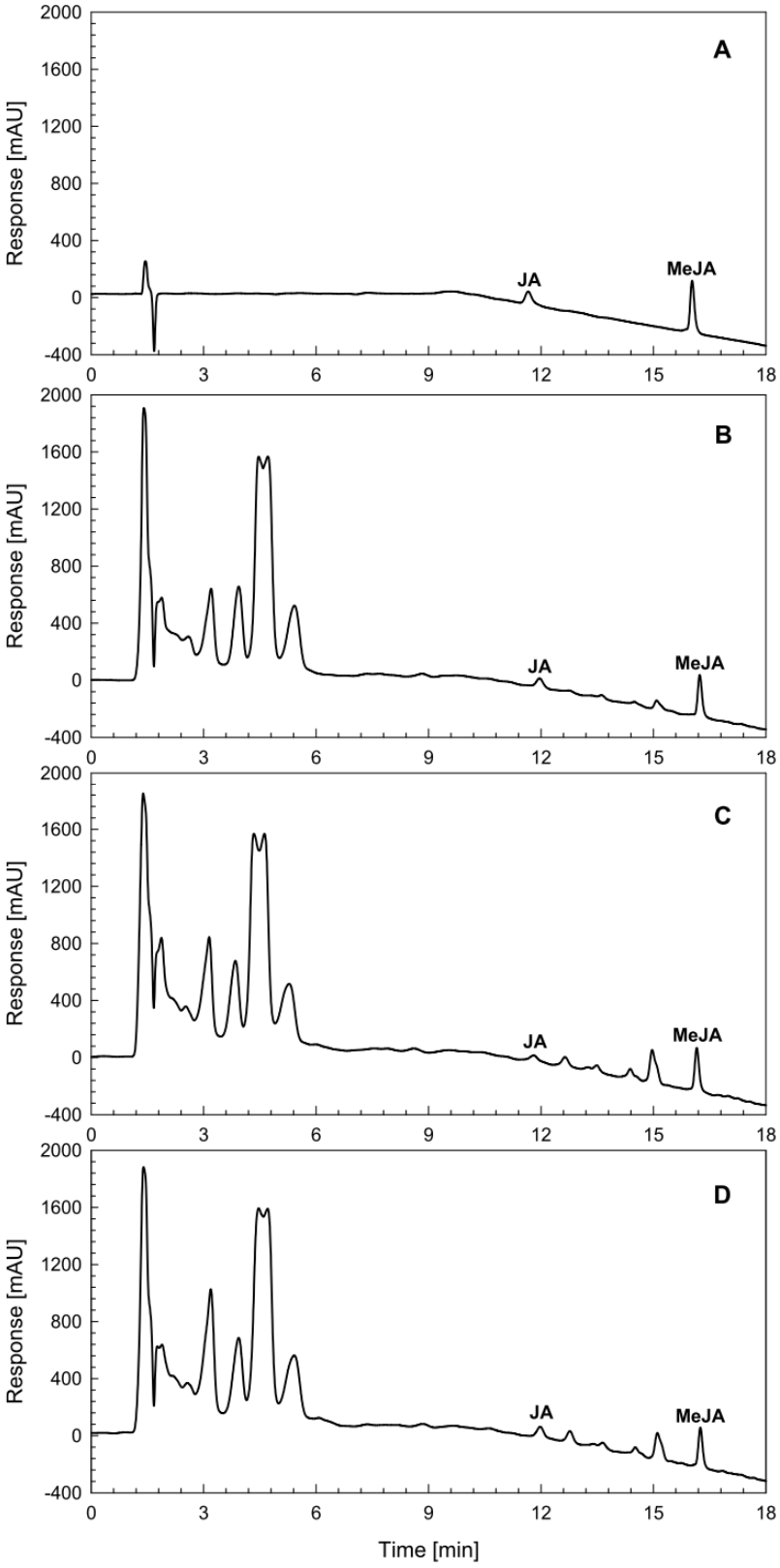


**Appendix S1.** Chromatographic analysis by RP-HPLC of jasmonic acid (JA) and methyl-JA (MeJA) standards (panel A), and soluble fractions from healthy (panel B), diseased (panel C), recovered (panel D) apple leaves, using MeJA as internal standard.

## *8.3 Characterization of lipolytic activities in green coffee* (Coffea arabica *L.*) *beans during storage*

### Introduction

In plants, the main site of triacylglycerol (TAG) storage is in the embryo and endosperm tissues of the seeds, depending on the species. TAGs form a hydrophobic matrix stored in specialized structures, named oil bodies, surrounded by a monolayer of phospholipids (Murphy, 2001). In germinating oilseeds, the mobilization of the stored fatty acid is essential to supply energy and carbon for embryonic growth, and lipolytic enzymes are responsible for the catalysis of the first step of lipid mobilization (Barros et al., 2010). Phospholipases (PLases) are a wide series of enzymes that hydrolyze phospholipids (PLs), and according to their sites of hydrolysis on PLs are classified into PLase D (PLD), C (PLC), A2 (PLA 2), A1 (PLA1), and B (PLB) (Wang, 2001). Multiple forms of phospholipases D, C, and A have been characterized in plants, and are involved in a broad range of functions in cellular regulation, lipid metabolism, membrane remodeling and signal transduction (Wang, 2001). The second class of enzymes are the TAG lipases, that hydrolyse TAGs at the oil/water interface to yield free fatty acids (FFAs) and glycerol (Quettier and Eastmond, 2009).

During coffee seed storage, lipolytic degradation promotes the production of metabolites that could be potentially harmful for seed viability and detrimental for the quality of green coffee beans. In fact, during prolonged storage, undesired changes within the lipid fraction may occur due to the oxidation processes, generating the so-called “off-notes” in the aroma of processed coffee (Speer and Kölling-Speer, 2006). Stored coffee beans are dried down to 12-13% of moisture content and since lipases are active at very low RH, even at less than 10% (Leopold, 1990), it is reasonable that some lipolytic enzyme could be active in commercial green coffee.

Up to date, little is known about lipolytic enzyme activities in green coffee beans. In this work the activity of phospholipases and lipases was measured in commercial green coffee beans harvested in different countries such as Ethiopia, India, Kenya and Tanzania.

## Materials and methods

### Plant material

Four commercial lots of *C. arabica* L. harvested in different countries were provided by Biolab, Illy Caffè, Trieste, Italy. The lots of coffee were characterized by different lipid content: Ethiopia, 14-15 %; India, 11 %; Kenya, 14-15 %; Tanzania, 16 %.

### Acetone powder preparation

Ten g of each coffee bean lot of was frozen in liquid nitrogen and ground in a blender (Ika Werke, Staufen, Germany) to obtain a fine powder. Then the powder was stirred for 4 h at 4 °C in 50 mL of chilling acetone (-20 °C), and subsequently centrifuged at 1900 **g** in a Sorvall RC-5B centrifuge (SS-34 rotor) for 15 min. The pellet was resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.4 M sucrose, homogenized with an Ultra Turrax (Ika Werke, Staufen, Germany) and finally centrifuged at 12,000 **g** in a Sorvall RC-5B centrifuge (SS-34 rotor) for 20 min. Supernatant was collected, filtered through a cotton gauze and stored at -80 °C.

### Assay of phospholipase $A_1$ and $A_2$ activities

The acetone powder, obtained from coffee seeds as previously described, was used to evaluate $PLA_1$ and $PLA_2$ activities. $PLA_1$ and $PLA_2$ activities were assayed fluorimetrically (Perkin Elmer spectrofluorimeter LS50) using two different probes: BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) was the fluorogenic substrate for PLA1 and PLA2, while PED6 (N-((6-(2,4-dinitrophenyl)amino)hexanoyl)- 2- (BODIPY - pentanoyl) - 1 - hexadecanoyl - sn - glycero - 3 - phosphoethanolamine, triethyl-ammonium salt) was used to measure $PLA_2$ activity. Both probes were solubilised in DMSO. Ten µg of protein was added to 1 mL of the assay buffer (50 mM Tris-HCl, pH 6.0, and 5 mM EDTA). The assay was carried out at 30 °C and started by the addition of 2 µL of BODIPY or PED6, to a final concentration of 2 µM. Fluorescence readings were recorded for 3 minutes (excitation wavelength set at 341 nm and emission wavelength set at 377 nm, respectively) and the enzymatic activity was expressed as arbitrary units. Furthermore, the effect of pH on $PLA_2$ activity was evaluated ranging from 5.5 to 8.5. Moreover, the modulation of $PLA_2$ activity was also monitored by increasing concentration of free calcium (ranging from 5 nM to 5 µM) in the presence of EGTA.

**Assay of lipase activity**

Lipase activity of coffee samples was assayed by a colorimetric method, using a kit from Randox (Lipase (LPS), Crumlin, United Kingdom). The kit contains a buffer (R1) and the chromogenic substrate 1,2-O-Dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester (DGGMR) that, when cleaved by the catalytic action of lipases, forms 1,2-o-dilauryl-rac-glycerol and an unstable intermediate, glutaric acid-(6-methyl resorufin) ester. The latter decomposes spontaneously in alkaline solution to form glutaric acid and methylresorufin. Being lipase activity proportional to the production of methylresorufin, the reaction was determined photometrically by a Multilabel plate reader at 570 nm (Perkin Elmer Wallac 1420 Victor3). The assay mixture was composed by 5 µl of extract (protein concentration 1.5 µg µl$^{-1}$), 155 µl of R1 and the reactions were started by the addition of 40 µl of DGGMR. The enzymatic activity was expressed as nmol methylresorufin mg$^{-1}$ protein min$^{-1}$, using an extinction coefficient $\varepsilon = 1.33 \times 10^5$ M$^{-1}$ cm$^{-1}$ at 570 nm for methylresorufin. Furthermore, the modulation of lipase activity was monitored by increasing concentration of EGTA and EDTA, up to 40 mM and 10 mM, respectively. Finally, the analysis of lipase activity inhibition was performed after pre-incubation of the samples for 30 min with tetrahydrolipstatin (THL) solubilised in DMSO, to a final concentration up to 300 µM.

**Protein determination**

Protein content of the samples was determined according to Bradford (1976), using BSA as a standard.

**Results & discussion**

The PLase activities measured fluorimetrically using BODIPY or PED6 probes showed similar results: in both cases, $PLA_1$ and $PLA_2$ or $PLA_2$ activities were higher in green coffee beans from Kenya (initial rate 3.76 A.U. sec$^{-1}$ and 5.68 A.U. sec$^{-1}$ respectively), while the lowest activity was detected in samples from India (initial rate 2.12 A.U. sec$^{-1}$ and 3.9 A.U. sec$^{-1}$ respectively) (Fig. 1).

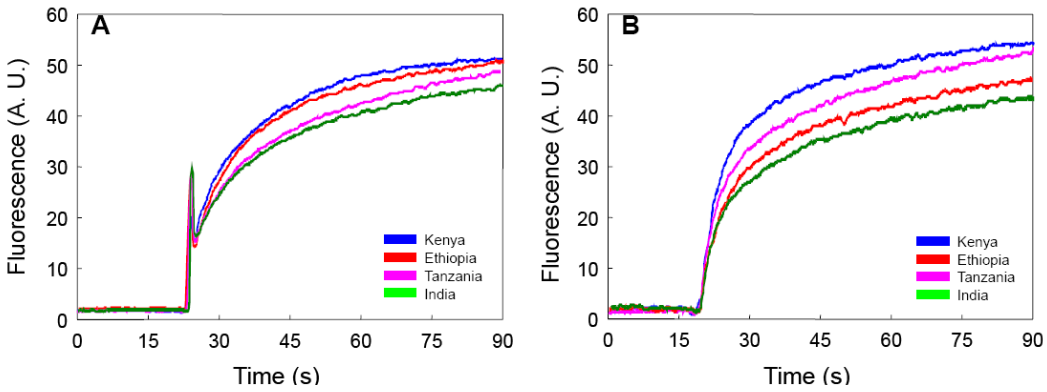


**Fig.1**. Phospholipase activity in green coffee beans harvested in different countries. Panel A: $PLA_1$ and $PLA_2$ activity assayed by BODIPY probe. Panel B: $PLA_2$ activity assayed by PED6 probe.

The pH dependence of $PLA_2$ activity was assayed in green coffee beans from Kenya and led to the identification of two main peaks, the first at pH 6.0 (initial rate 3.1 A.U.min$^{-1}$) and the second at pH 7.5 (initial rate 3.3 A.U.min$^{-1}$) (Fig. 2). The characterization of phospholipase A showed that the enzyme activity had two peaks, at pH 6.0 and 7.5, suggesting the presence of at least two isoenzymes.

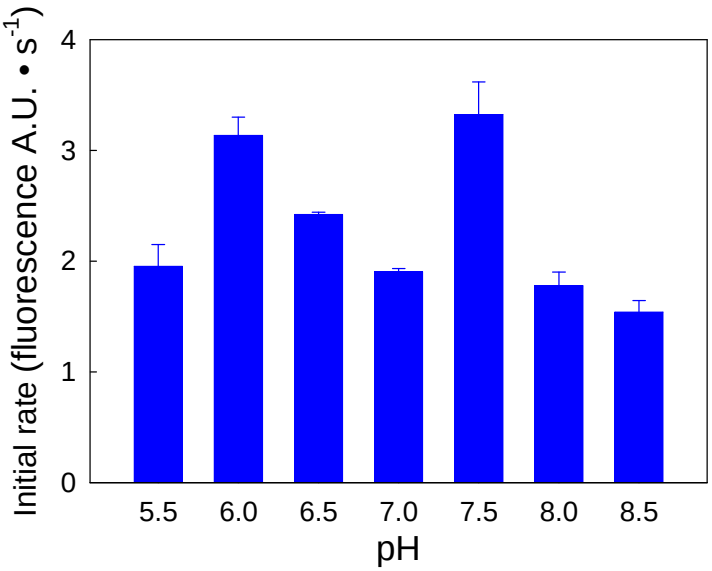


**Fig.2** Effect of pH on $PLA_2$ activity in green coffee beans harvested in Kenya.

Since citosolyc $PLA_2$ is stimulated by $Ca^{2+}$ (Dessen, 2000), the modulation of $PLA_2$ activity by increasing concentration of free calcium was performed. As shown in Fig. 3, $PLA_2$ activity in coffee beans from Kenya, Ethiopia, India and Tanzania was not stimulated by calcium ions but was instead slightly decreased at pH 6.0 by increasing concentrations of free calcium (Panel A). This effect was more marked at pH 7.5, where $PLA_2$ activity was strongly inhibited starting from 50 nM $Ca^{2+}$ (panel B). Therefore, this phospholipase activity could be ascribed to intracellular $Ca^{2+}$-indipendent $PLA_2$ (Wang, 2001).

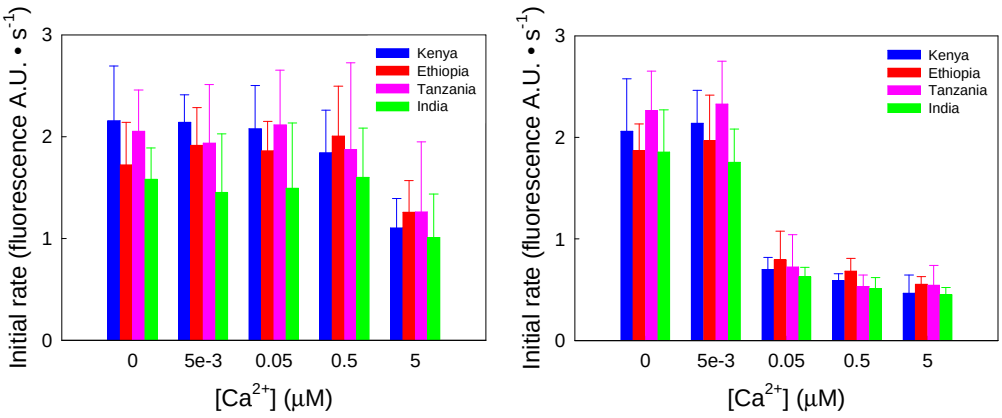


**Fig. 3**. $Ca^{2+}$ modulation of $PLA_2$ activity. Panel A, $PLA_2$ activity assayed at pH 6.0. Panel B, $PLA_2$ activity assayed at pH 7.5.

In order to better characterize the lipolytic activity in green coffee beans, lipase activity was also assayed in the four lots. Lipase activity, measured in the green coffee beans from Kenya, Ethiopia, India and Tanzania, did not show significant differences among the samples (Fig. 4). Therefore, the further characterization of the lipase activity was performed on green coffee beans from Kenya.

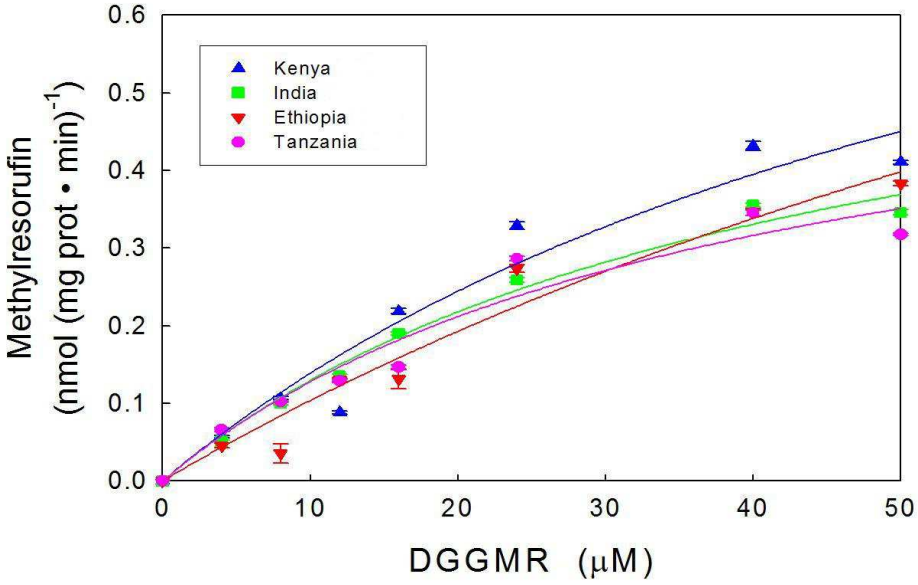


**Fig. 4**. Lipase acitivity in green coffee beans harvested in different countries.

In literature, evidence about the stimulation of lipase activiy by bivalent ions, and in particular calcium, has frequently been reported in oilseed species, such as lupin (*Lupinus luteus* L.), almond (*Amygdalus communis* L.), Africa bean seed (*Pentaclethra macrophylla Benth*), castor bean seed (*Phaseolus vulgaris*), rapeseed (*Brassica napus* L.), barbados nut (*Jatropha curcas* L.), French peanut (*Panchira aquatica Bombacaceae*), laurel seed (*Laurus nobilis* L.), oat seed (*Avena fatua*) (Barros et al., 2010). Therefore, the analysis of lipase activity by increasing concentration of EGTA, a $Ca^{2+}$ chelant, was performed. The data obtained revealed that increasing concentration of EGTA inhibited lipase activity: at the higest concentration (40 mM), lipase activity was inhibited of about 50 % (Fig. 5), while EDTA (a chelating agent of bivalent ions) was inhibitory only at the higest concentration (10 mM) (data not shown).

These results indicate that lipase activity in green coffee is calcium-dependent, similarly to what observed in many oilseed species (Barros et al., 2010).

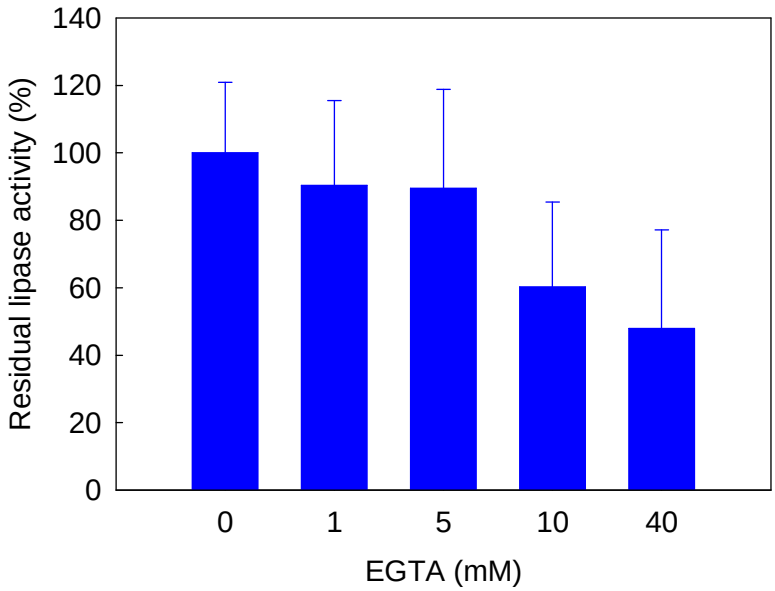


**Fig. 5**. Effect of EGTA on lipase activity in green coffee beans harvested in Kenya.

Finally, lipase activity was characterized using THL, a selective lipase inhibitor interacting with a serine present in the active site of lipases (Hadvary et al., 1988). Increasing concentration of THL strongly inhibited lipase activity, which reached about 50 % at 300 µM (Fig. 6). These results are in agreement with inhibition of lipase activity performed with oil palm fruit (Ebonogue et al., 2006), *Chromobacterium viscosum* and *Rhizopus oryzae* (Potthoff et al., 1998)

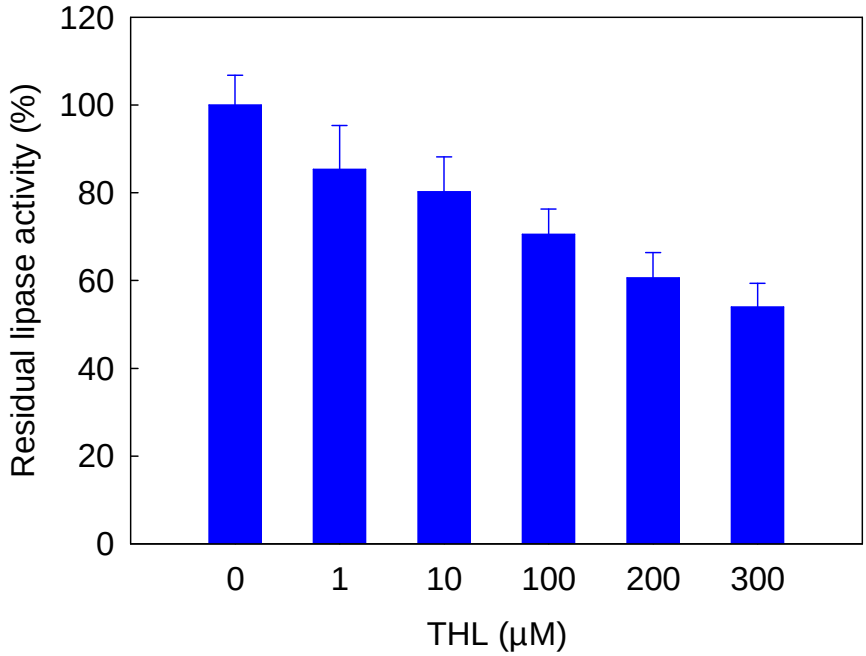


**Fig. 6.** Inhibition induced by increasing concentrations of THL on lipase activity in green coffee beans harvested in Kenya.

## Conclusions

These findings show that commercial green coffee beans exhibit lipolytic activities that consequently could degrade lipid bodies and release FFAs in the endosperm tissues, according to what observed in ageing coffee seeds by Dussert and coworkers (2006). Commercial green coffee is normally dried and stored for several months, but it loses embryo viability during such processing methods because of its behaviour as an intermediate seed. In this contest, FFAs are not all metabolized via β-oxidation and glyoxylate cycle, as it would happen if the embryo is viable; rather, FFAs could be used as substrates for the oxylipin pathway and undergo free radical attacks, leading to dangerous lipid derivatives and undesired products such as saturated aldehydes (e.g. hexanal). Being TAGs essential precursors for the aroma of roasted coffee beans, the alterations of lipid fraction can seriously impair the final coffee quality.

Even if the four lots of coffee seeds (Ethiopia, India, Kenya and Tanzania) used for the study were characterized by different lipid content, lipase and phospholipase A activity resulted very similar among the samples. Hence, it could be assumed that in commercial green coffee seeds is present a common pattern of lipolytic enzymes, which are potentially harmful for the quality of the final product.

These initial characterization of lipolytic activities would therefore help to clarify the biochemical processes occurring in coffee beans during storage, leading to the identification of the correct strategies for preserving green coffee quality. In particular, since neither seed provenance (and consequently cultivation practices), nor plant cultivar, nor lipid content, affect lipolytic activities, it can be stated that post-harvest treatment, e.g. wet or dry processes, and storage conditions are crucial to maintain oxidative processes under control, preserving seed viability and final quality.

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## *8.4 Lipase activity and antioxidant capacity in green coffee* (Coffea arabica *L.*) *during germination*

**Introduction**

Coffee seeds (*Coffea arabica* L.) are classified as intermediate seeds (Ellis, 1990), since their water content in ripe fruit is up to 52 %, depending on post-harvest procedures of coffee beans, i.e. wet and dry processing (Wintgens, 2004). Generally, intermediate seeds can tolerate desiccation to moisture contents of about 7 to 12 %, depending on the species, but further drying leads to rapid loss in viability, and damage could occur on further desiccation (Ellis et al., 1990; Hong and Ellis, 1996).

Green coffee seeds represent vital and living organs, where various metabolic reactions take place during processing, e.g. germination-related metabolism and stress metabolism (Bytof et al., 2007). In particular, the intermediate and recalcitrant seeds are characterized by the lack of dormancy and partial desiccation, features that are associated to an appreciable metabolism and make the seeds ready to germinate. The germination-related metabolism has been monitored in green coffee by the expression of germination-specific enzymes (i.e. isocitrate lyase) and the resumption of cell cycle activity, evaluated as ß-tubulin accumulation (Selmar et al., 2006). The extent and time course of this metabolism depends strongly on post-harvest processing. Indeed, germination-related reactions are activated earlier during wet processes in comparison to dry ones (Selmar et al., 2006; Bytof et al., 2007). As a consequence of the features described above, the storage of coffee beans depends on the result of various and complex effectors, acting at different times.

The products of germination-related metabolism of green coffee could have a negative impact in commercial beans, through the deterioration of well-known precursors of coffee flavour, such as free amino acids and free sugars, which could lead to a poor final cup quality. The so-called off-notes that occur during green coffee storage are also caused by undesired changes within the lipid fraction due to oxidation processes (Speer and Kölling-Speer, 2006; Selmar et al., 2008). In order to study the biochemical and physiological bases of green coffee storage behaviour, Dussert and co-workers (2006) demonstrated that oxidative stress, lipid hydrolysis, phospholipid loss and decrease of the concentrations of two crucial antioxidant compounds, such as glutathione and ascorbate, are involved in the ageing of coffee seeds stored at 20 °C and at intermediate relative humidity (81 %).

Therefore, the control of degenerative processes is an essential aspect not only for storage but also for germination of coffee seed, since the embryo viability could be affected

by the uncontrolled release of free fatty acids. In particular, the lipid fraction in green coffee represents a significant part of the seed, ranging from 13 to 17 % of dry matter, consisting mainly of triacilglycerols (Speer and Kölling-Speer, 2006), whose composition determines the generation of compounds that are responsible for the formation of aroma in roasted bean (Joet et al., 2010). Since the lipid fraction is the preferred substrate for degradation reactions (Speer and Kölling-Speer, 2006), the content of free fatty acids (FFA) is correlated to increase temperature, oxygen content and moisture, after storage for 18 months (Speer and Kölling-Speer, 2006). The role of lipid hydrolysis in the behaviour of coffee seed during storage has been further confirmed by the negative correlation observed between coffee seed viability and FFA content (Laffargue et al., 2007). Nevertheless, it is yet not clear how storage condition could affect lipid metabolism and degradation during storage and the initial phases of germination.

The removal of the pericarp of the coffee cherry represents a post-harvest treatment that could further influence seed physiology. In wet process, the pericarp is mechanically depulped and the residues are removed after a fermentation step. The green coffee beans so obtained are still covered by the parchment, the fruit endocarp, and remain viable far longer than hulled beans obtained by dry process. As a consequence, this maintenance of viability has a positive effect on the preservation of green coffee quality during prolonged storage (Selmar et al., 2008).

Coffee is an economically relevant commodity, internationally traded as green coffee, but the knowledge of its lipid metabolism during storage and germination is still very scarce. To our knowledge, this is the first characterization of lipase activity in coffee bean in both seeds and seedlings in the presence or absence of the parchment. In addition, the antioxidant properties in germinating green coffee beans have been analysed, aiming to identify the possible correlation between the antioxidant protection and the lipid degradation processes.

## Materials and methods

### Plant material and seed germination

Green coffee beans (*C. arabica* L.) harvested in Colombia were provided by Illy Caffè, Trieste, Italy. The seeds were divided into two lots, with (+) and without (-) parchment, and kept in water for 7 days at 28 °C in the dark. After imbibition, the seeds were sown in perlite at 28 °C in the dark and daily watered. At each sampling day (0, 3, 7, 10, 12, 15, 18,

21, 24, 28 days after imbibition, DAI), about 40 seeds were collected, frozen in liquid nitrogen and stored at -80 °C.

**Acetone powder preparation**

Ten g of frozen coffee beans was ground by a blender (Ika Werke, Staufen, Germany) to obtain a fine powder. Then, the powder was stirred for 4 h at 4 °C in 50 mL of chilling acetone (-20 °C), and subsequently centrifuged at 1900 *g* (SS-34 rotor) for 15 min. The pellet was dried under nitrogen, resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.4 M sucrose, homogenized with an Ultra-Turrax (Ika Werke, Staufen, Germany), and finally centrifuged at 11,900 *g* (SS-34 rotor) for 20 min. The supernatant was filtered through a cotton gauze and stored at -80 °C.

**Assay of lipase activity**

Lipase activity was assayed by a colorimetric method, using a kit from Randox (Lipase, Crumlin, UK). The assay was based on the hydrolysis of a specific substrate (1,2-O-Dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester) (DGGMR): this chromogenic compound is cleaved by the catalytic action of lipases into 1,2-o-dilauryl-rac-glycerol and glutaric acid-(6-methyl resorufin) ester, an unstable intermediate. The latter decomposes spontaneously in alkaline solution to form glutaric acid and methylresorufin, a coloured compund that was determined photometrically by a Multilabel plate reader at 570 nm (Perkin Elmer Wallac 1420 Victor3). The reaction mixture was composed by 5 µl (approx. 7.5 µg protein) of extract, 155 µl of buffer and the reactions were started by the addition of 40 µl of DGGMR at 30 °C. The enzymatic activity was expressed as nmol methylresorufin $mg^{-1}$ protein $min^{-1}$, using an extinction coefficient $\varepsilon = 1.33 \times 10^5$ $M^{-1}$ $cm^{-1}$ at 570 nm for methylresorufin.

The analysis of inhibition of lipase activity was performed using tetrahydrolipstatin (THL) and an anti-lipase antibody (Ab) raised against the lipase of castor bean (*Ricinus communis* L.) (Agrisera, Cernusco sul Naviglio, Milan, Italy). THL and anti-lipase Ab were added to the reaction mixture at different concentrations (up to 300 µM and 3 ng $ml^{-1}$, respectively) and incubated for 30 min at 30 °C.

**Antioxidant capacity assay**

The antioxidant capacity of germinating coffee seeds was evaluated by crocin kinetic competition test as described by Tubaro and coworkers (Tubaro et al., 1996). This test was

performed on the hydrophobic compounds extracted with 1,4-dioxane from 1 g of ground material obtained from germinating coffee seeds at different sampling days. Then, the samples were centrifuged at 7600 *g* (SS-34 rotor) for 20 min and the supernatant was collected. Peroxy radicals were generated *in situ* by diazo compound decomposition. The bleaching of crocin was directly correlated to radical production and occurred with a constant speed. When part of the peroxyl radicals was quenched by other antioxidants, the bleaching rate was lower, and was correlated to the concentration of the antioxidants present in the sample. The ratio between the bleaching speed of crocin, with or without other antioxidants, yielded the whole antioxidant capacity of the sample. The slope of a known antioxidant, such as α-tocopherol, was calculated as reference. The results were expressed as millimolar equivalents of α-tocopherol $mg^{-1}$ fresh weight.

**SDS-PAGE and Western Blotting**

Proteins from acetone powder preparation (about 20 µg) were separated by 10 % (w/v) SDS-PAGE, under reducing conditions and the gels were stained with Coomassie Brilliant Blue R-250. Immunoblotting was performed according to standard techniques, using the anti-lipase Ab at 1:20,000 dilution. The immune reaction was detected with an antirabbit IgG alkaline phosphatase-conjugated secondary Ab (1:2500 dilution), followed by the addition of the substrate 5-bromo-4-chloro3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT), buffered by substrate tablets (Sigma–Aldrich, Milan, Italy). Computer-assisted densitometric analysis of the Western blot was performed by Quantity One Software (Bio-Rad, Hercules, CA, USA).

**Gas chromatography analisys**

Two g of frozen coffee beans was ground by a blender (Ika Werke, Staufen, Germany) to obtain a fine powder. The powder was stirred for 2 h at 4 °C in 10 ml of exane-ether (1:1) and the organic phase was dried with anhydrous sodium sulphate (0.1 g $ml^{-1}$) and finally filtered with Whatman paper (Maidstone, England). The sample was dried under nitrogen. About 100 mg of oil was added to 1 ml of internal standard solution containing 1 mg methyl heptadecanoate and 1 mg triheptadecanoin. Then 20 - 30 µl of solution was dried under nitrogen stream and solubilised in 200 µl of silylation reagent; finally, it was dried by a soft nitrogen flow and resuspended in 2 ml of n-heptane. One µl was injected for GC analyses, performed using a Therm Fisher capillary gas chromatography system with cold on column injection port with flame ionisation detection and a capillary column, fused silica, (6 m x 0.32

mm x 0.10 - 0.15 µm). The analyses were carried out in programme temperature mode from 80 °C to 220 °C at 20 °C $min^{-1}$ and then from 220 °C to 340 °C at 5 °C $min^{-1}$. The carrier gas was helium, auxiliary gases: hydrogen (flow rate: 25 ml $min^{-1}$), air (flow rate: 300 ml $min^{-1}$) (Draft presented by Chemical Expert Group, International Olive Council, Madrid, 2010).

**Protein determination**

Protein content of the samples was determined according to Bradford (1976), using BSA as a standard.

## Results

### Characterization of lipase activity in green coffee seeds

Lipase activity was evaluated in green coffee beans from Colombia at 12 DAI, just before radicle protusion in seeds without parchment. The activity was detected with increasing concentration of DGGMR, ranging from 0 to 100 µM (Fig. 1). The initial rate values fitted the Michaelis-Menten equation, whose kinetic parameters were: $V_{max}$ = 0.23 ± 0.01 µmol $min^{-1}$ $mg^{-1}$ protein and $K_M$ = 14.18 ± 2.79 µM.

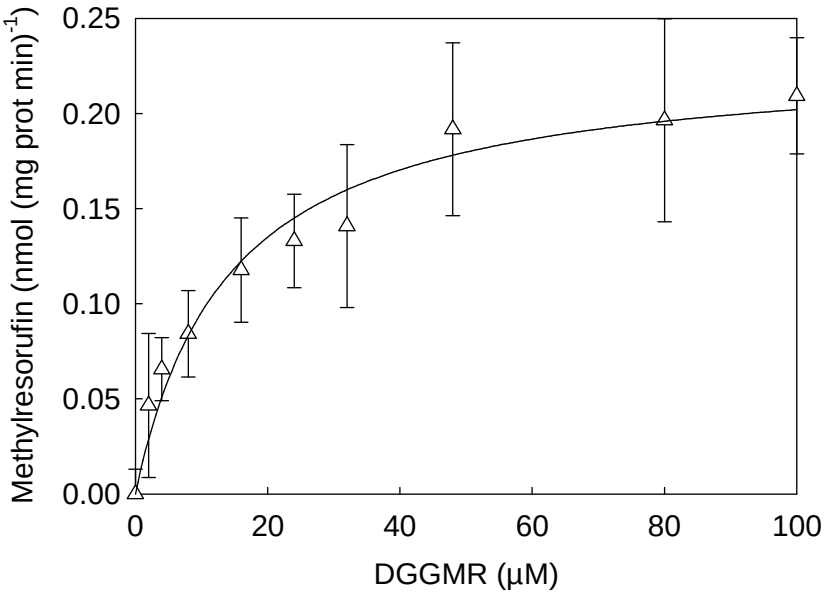


**Fig. 1.** Characterization of lipase activity in green coffee seeds at 12 DAI (△). The initial rate of lipase activity on DGGMR concentration fitted ($r^2$ = 0.97) the equation $V = V_{max}[DGGMR]/(K_M[DGGMR])$. Data (n = 3) are means ± S.D.

To further characterize the lipase activity, two inhibition assays of the lipase activity were performed on coffee seeds without parchment at 12 DAI. The lipase activity was strongly decreased by the specific lipase inhibitor THL, which reacts with a serine present in the active site of lipases (Hadvary et al., 1988), reaching approx. 67 % inhibition at 300 µM (Fig. 2).

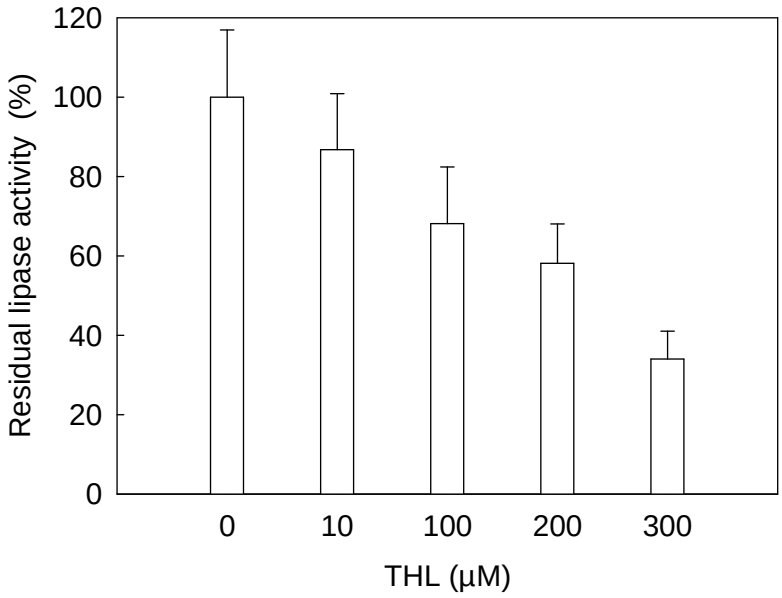


**Fig. 2.** THL inhibition of lipase activity in green coffee seeds at 12 DAI. The initial rate was expressed as percentage with respect to the control (without THL). Data (n = 3) are means ± S.D.

Moreover, an immunological approach was carried out using a polyclonal anti-lipase Ab. Figure 3 shows that in green coffee beans the anti-lipase Ab inhibited lipase activity in a concentration-dependent manner, following the equation $y = y_0 + a*e^{(-b*x)}$ and showing a maximum inhibition of approx. 20 % in the presence of 0.6 ng Ab.

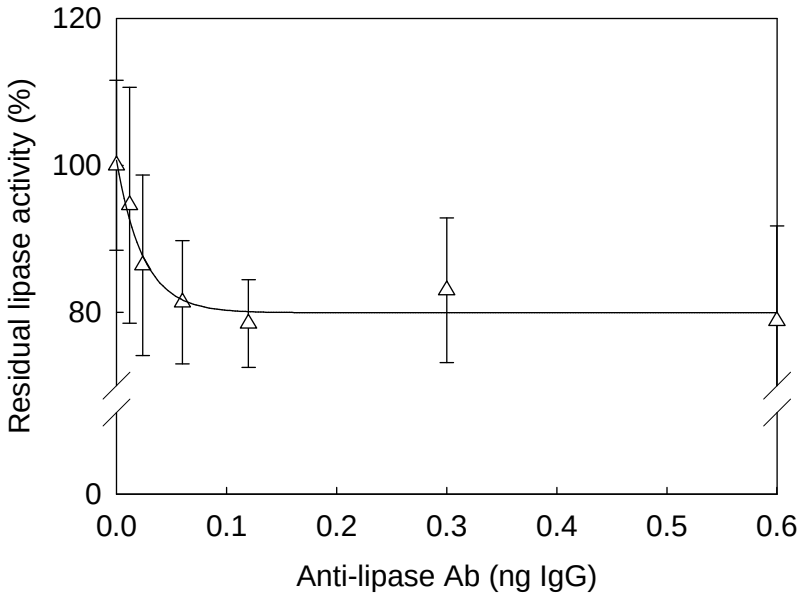


**Fig. 3.** Inhibition induced by increasing concentrations of anti-lipase Ab on lipase activity in green coffee seeds at 12 DAI (△). The initial rate was expressed as percentage with respect to the control (without anti-lipase Ab). Data (n = 3) are means ± S.D. Data fitted ($r^2$ = 0.95) to an exponential decay function, two parameter equation $y = y_0 + a*e^{(-b*x)}$, where the parameters found were: $y_0$ = 79.9 ± 1.2, the residual relative lipase activity, a = 20.8 ± 2.4, the fraction of relative lipase activity that is amenable to inhibition by the antibody; b = 61.8 ± 17.1, the rate of decline of relative lipase activity with increasing concentrations of the antibody.

**Influence of parchment on germination and lipase activity of green coffee seeds**

The developmental stages occurring during germination in coffee seeds are schematically shown in figure 4 panel A. Green coffee seeds sown without parchment showed a visible radicle protuberance at 7 DAI, indicating the embryo growth inside the endosperm. The radicle emission appeared at about 15 DAI and in the following days the radicle elongation was noted. On the contrary, the presence of the parchment in green coffee seeds strongly delayed the radicle emission: in fact, this event occurred approximately at 28 DAI.

During germination, lipase activity was measured in green coffee seeds with or without parchment up to 28 DAI (Fig. 4, panel B). Lipase activity was very similar for seeds with or without parchment in the first three sampling days (0, 4 and 8 DAI) and the values were not significantly different. From 10 DAI, the enzymatic activity showed a different pattern: green coffee seeds with parchment were characterized by a slow continuous increase of lipase activity, with the highest value observed at 28 DAI. Conversely, green coffee seeds without parchment showed a peak of lipase activity at 12 DAI, followed by a decrease.

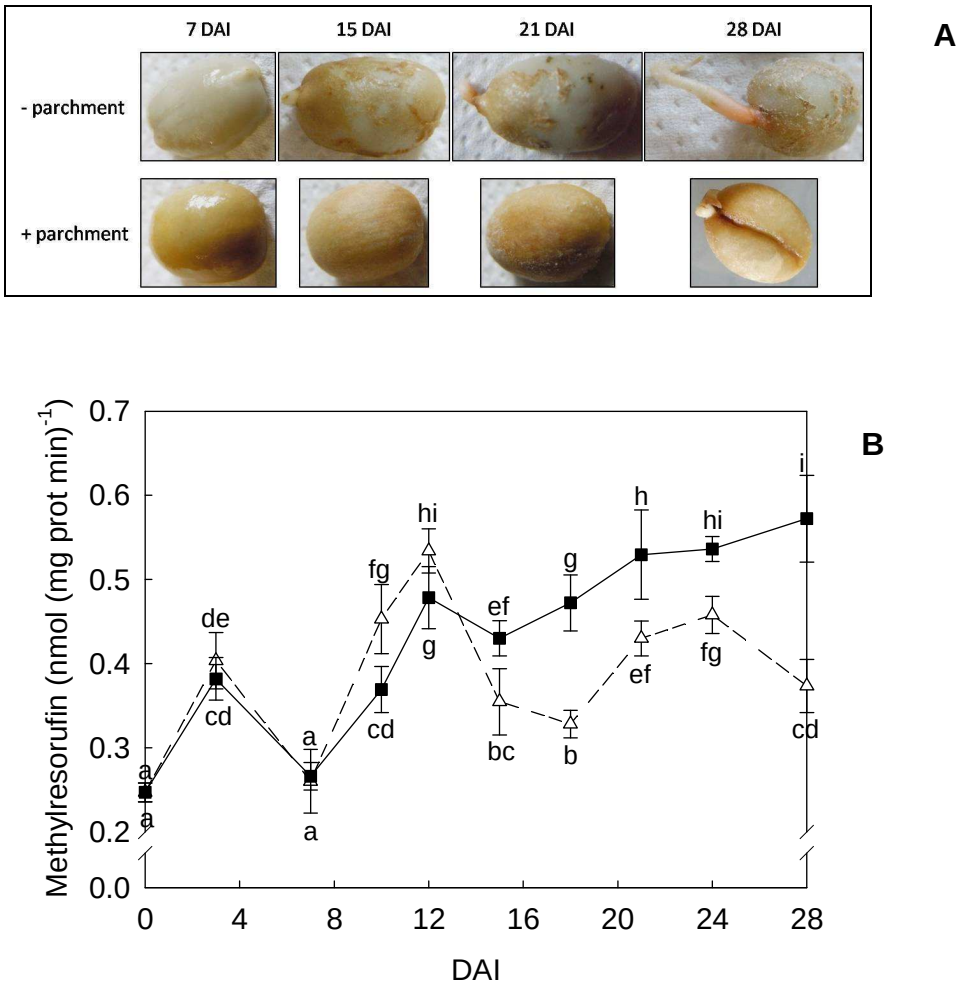


**Fig. 4.** Influence of parchment on lipase activity at germinating green coffee seeds. Panel A: morphological development of germinating green coffee seeds with (+) and without (-) parchment. Panel B: lipase activity in green coffee seeds with (■) or without (△) parchment. Data (n = 3) are means of the initial rate of lipase activity ± S.D.

This pattern was supported by the immunoblotting assay performed using the anti-lipase Ab: in green coffee seeds, either without (Fig. 5, panel A) or with (Fig. 5, panel B) parchment, the anti-lipase Ab cross-reacted with a protein of approx. 60 kDa. The intensity of this cross-reaction, obtained from the densitometric analysis of the immunoblot membrane, appeared different during the germination stages in both seed lots (Fig. 5, panel C).

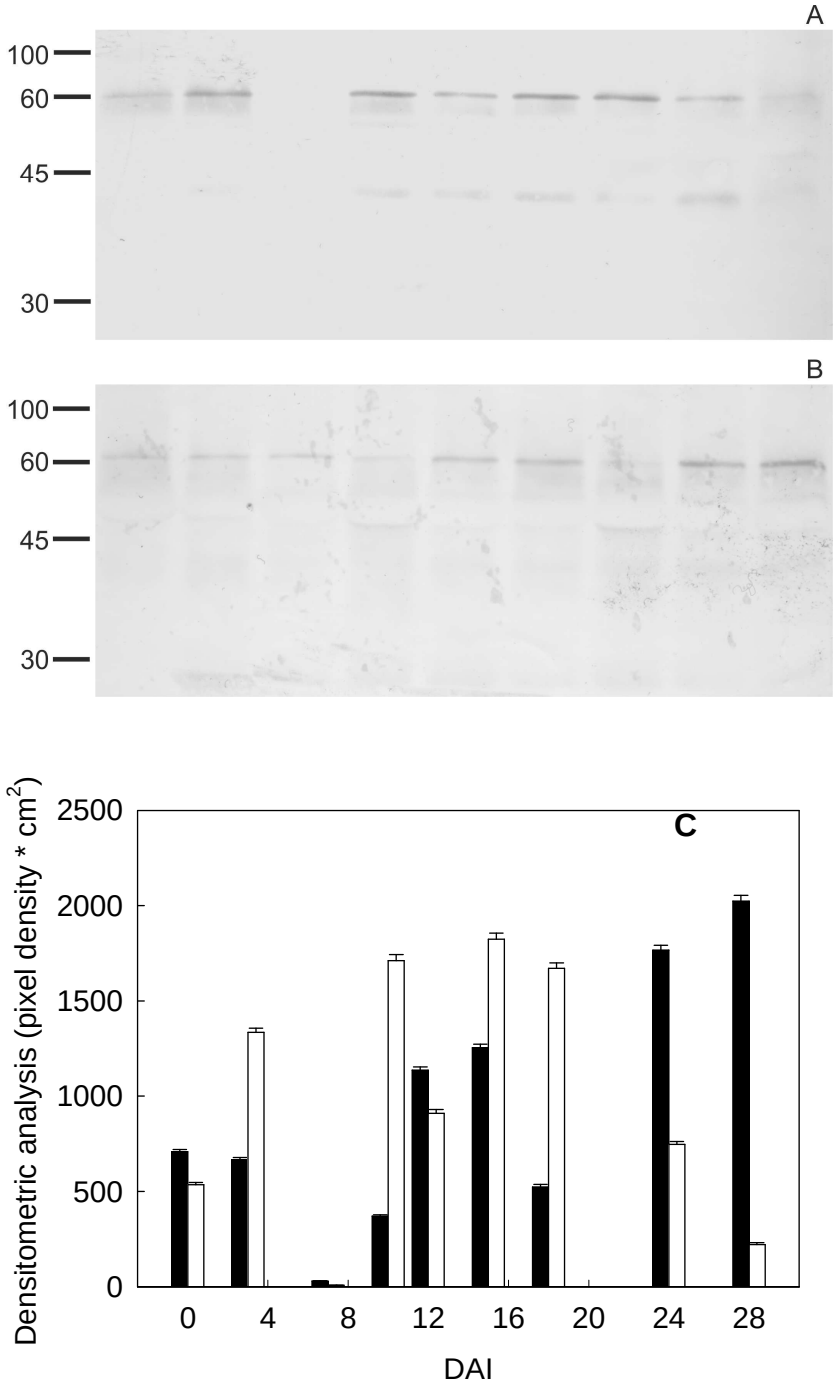


**Fig. 5.** Immunoblot analyses of lipase from green coffee seeds at different DAI. Western blots of proteins (approx. 20 µg) obtained from green coffee seeds without (Panel A) or with (Panel B) parchment. The values represent the apparent molecular mass of molecular standards. Panel C: densitometric analysis of immunoblot. Data (n = 3) are mean values ± SD, calculated from three independent immunoblotting experiments. Columns represent the spot density from either green coffee seeds with (black) or without (white) parchment.

In green coffee seeds without parchment, the lipase increased during the first sampling days, exhibiting its maximum at 15 DAI, to decrease hereafter. A different pattern was observed in green coffee seeds with the parchment, where the lipase was present with an increasing pattern, reaching the highest level at 28 DAI. The patterns of cross-reactivity of coffee seed lipase with the anti-lipase Ab were therefore correlable with those of enzymatic activity shown in Fig. 4, panel B.

Aiming to describe the degree of oxidative status of green coffee seeds with or without parchment, the antioxidant capacity of coffee beans was measured during germination (0, 7, 12, 21, 28 DAI) (Fig. 6). The pattern exhibited a trend that resembled the one described for lipase activity: at 0 and 7 DAI, total antioxidant capacity resulted very similar for seeds with or without the parchment. During the following germination steps, the lipophilic antioxidant activity in seeds without parchment reached a peak at 12 DAI to decrease later, while in the seeds with parchment the maximum was reached with a delay, showing the highest total antioxidant capacity at 21 DAI.

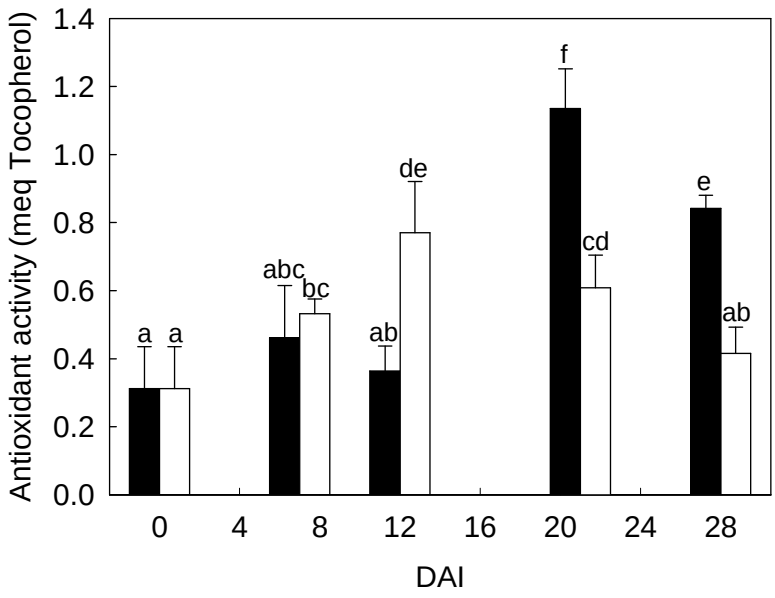


**Fig. 6.** Lipophilic antioxidant capacity in green coffee seeds at different DAI. Antioxidant capacity was measured either in green coffee seeds with (black columns) or without (white columns) parchment. Data (n = 3) are means ± S.D.

**FFA analysis**

The presence of FFA in coffee oil, obtained as above reported, has been measured throughout germination. Figure 7, panel A shows a typical gas-chromatogram from coffee oil after extraction with exane/ether. The content of FFA was evaluated in germinating seeds

with or without parchment (Fig. 7, panel B). In green coffee seeds with the parchment, the accumulation of FFA slightly increased, excepting for a little decrease observed for 21 DAI. In green coffee seeds without the parchment, FFA content increased at 7 DAI, then it decreased at 14 DAI. Finally, FFA analysis showed a rapid and sharp increase at 28 DAI. FFA profile was not correlated with the antioxidant capacity.

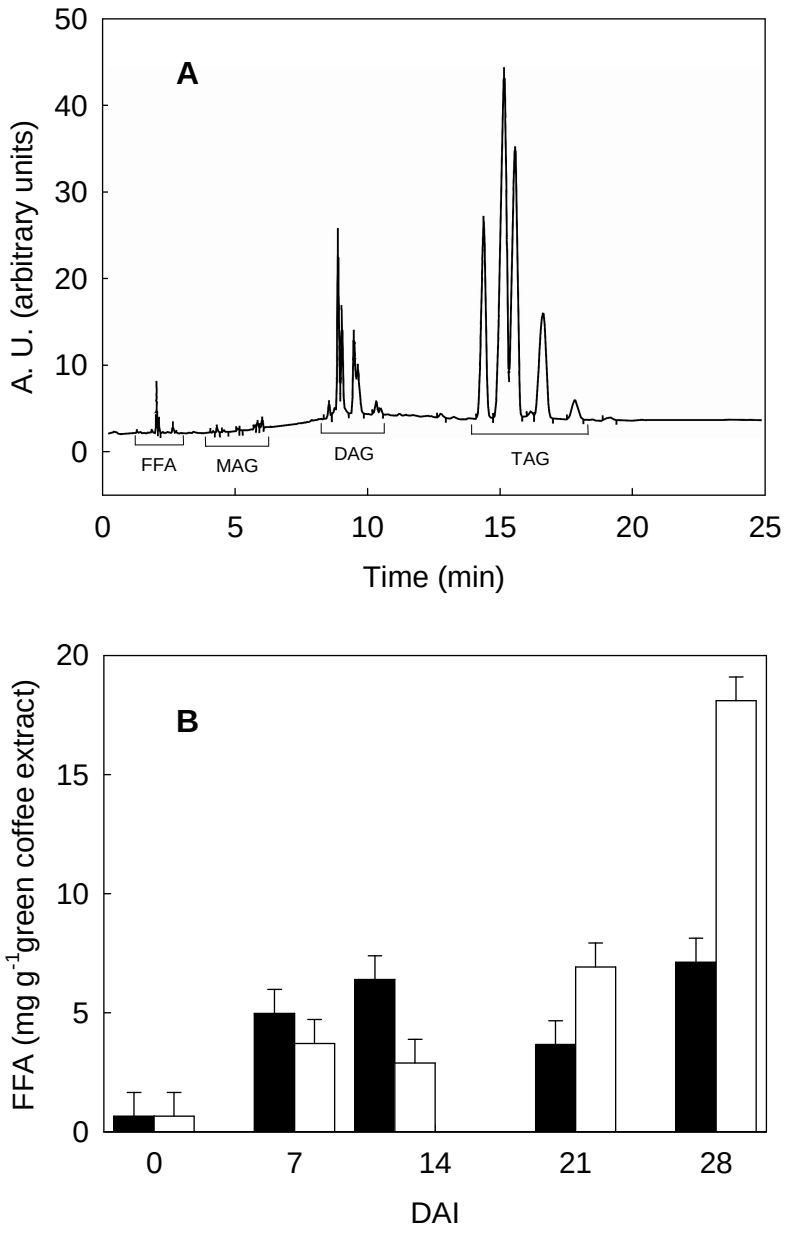


**Fig. 7**. Analyses of FFA in green coffee seeds at different DAI. Panel A: Typical gas-chromatography analysis of green coffee oil after extraction with exane/ether after. Panel B: FFA were detected either in green coffee seeds with (black columns) or without (white columns) parchment.

**Discussion**

Lipid bodies in oilseeds are mainly composed by triacylglycerols and in coffee seeds they are generally located in the endosperm (Speer and Kölling-Speer, 2006). Triacylglycerols are metabolized during storage, leading also to the production of undesired compounds responsible for a decrease of the final cup quality or to the impairment of germination in propagation material (Selmar et al., 2008). The release of free fatty acids could, indeed, trigger either degenerative processes, resulting in lipid oxidation/peroxidation, or fuelling high-energy demanding processes, e.g. respiration and sugar synthesis for embryonic growth (Quettier and Eastmond, 2009). Even though lipase activity in coffee seed represents a crucial step during storage and germination, few studies have described and characterized it (Kurzrock et al., 2005). Therefore, this paper represents, to our knowledge, the first biochemical characterization of lipases from coffee bean.

The lipase activity in coffee beans was characterized by kinetics parameters ($K_M$ and $V_{max}$, Fig. 1), that were similar to what reported for lipases from Barbados nut (*Jatropha curcas* L.), whose seeds are classified as recalcitrant (Staubmann et al., 1999), but different to those found in sunflower seeds, French peanut (*Panchira aquatica*), wheat, oat (Barros et al., 2010), rice (Vijayakumar and Gowda, 2012) and rape (Belguith et al., 2009) seedlings. The higher affinity for the substrates, exhibited by coffee lipases, suggests that in such seeds, containing a high amount of stored lipids, the lipolytic activities, and in particular lipases, could play a regulative role during the first steps of germination. In addition, the presence of coffee lipase activity was confirmed in germinating coffee seed by the inhibition by THL (Fig. 2), a selective and irreversible inhibitor of lipases, and by a polyclonal antibody raised against purified alkaline lipase from castor (*Ricinus communis*) beans (Fig. 3). This antibody cross-reacted with a protein of approx. 60 kDa (Fig. 5), a value similar to that of the acid lipase RcOBL1 isolated and characterized in oil body membranes from castor bean (Eastmond, 2004).

In green coffee the lipase activity was present in seed before imbibition and further induced during the germination process, which followed a biphasic behavior similar in both seeds with or without parchment (Fig. 4, panel B), even though the phenomenon showed a delay in the former. In particular, in coffee seeds without parchment, a sharp second peak was observed after 12 DAI. On the contrary, the lipase activity gradually increased in coffee with parchment, in parallel with the delay in germination, indicating a generally slowed metabolism in such seeds, in agreement to previous observations (Selmar et al., 2008).

A similar trend was also observed by the Western blot analysis of total coffee bean proteins, where the detection of lipases was possible since the very first DAI, but then was very low at around 8 DAI, to rise again after about 12 until 28 DAI in parchment coffee seeds (Fig. 5). The biphasic activity, being also confirmed by the expression pattern obtained by Western blot analysis (Fig. 5), could be attributable possibly to *ex novo* synthesis or to different lipase isoenzymes, similarly to what found in rice (Vijayakumar and Gowda, 2012). The delay in activation of germination and root development can be explained by the flattening of metabolic activity induced by the parchment, which lowers gas exchange. Therefore, it is possible to speculate that lipase activity would be exerted firstly by enzymes already present in coffee beans, and subsequently, during the second phase, by *ex novo* synthesized lipases or different isoenzymes induced by the germination process.

According to the above reported observations, the analysis of antioxidant capacity indicates that the parchment was again crucial for preventing the oxidation of the lipophilic fraction, being the parchment seeds less prone to oxidation (Fig. 6). Indeed, the parchment could act as a barrier to the oxygen fluxes towards the embryo/endosperm, thus limiting the production of ROS. This situation allows the germinating seeds to control the ROS level within the "oxidative window" for germination, as proposed by Bailly and coworkers (2008). In addition, the release of FFA, as expected, increased in both samples reaching a very high value in coffee seeds without parchment (Fig. 7, panel B). This result would depend on the initial higher lipase activity observed until 12 DAI (Fig. 4, panel B). Nevertheless, the accumulation of FFA at 28 DAI would hinder seeds to metabolize all FFA by β-oxidation, leading instead to lipid oxidation and to a decreased antioxidant capacity in seeds without parchment (Fig. 6).

Although the oxidative signals, mainly represented by ROS, are involved in seed germination, their concentration must be adequate to allow the completion of this process (Bailly et al., 2008). Nevertheless, ROS excess is detrimental and a delicate balance is needed to prevent the activation of a high oxidative metabolism, which is a harmful feature and causes a rapid decline of embryo (Pasquini et al., 2012).

In conclusion, our data suggest that coffee beans possess lipase activity that is still present during storage and is increased during germination. The lack of quiescence and ABA sensitivity of intermediate coffee seed requires a more strict metabolic control of all the degradation processes, necessary to provide new organic skeleton for the development of the seedling. In particular, a fine tuning is necessary to avoid the uncontrolled release of ROS, especially in seed exhibiting high lipid content in the endosperm. Therefore, we propose that

in coffee seeds the regulation of the gas exchange exerted by the parchment would be considered similar to what demonstrated in holm oak seed, where plastic films limited the gaseous exchange, prolonging the dormant-like state and consequently seed viability (Pasquini et al., 2012). In particular, the decrease of oxygen permeation due to the parchment in coffee seeds could lead to positive effects during storage and germination, e.g. through the decrease of both degeneration products and oxidative processes. Hence, our data supply a physiological explanation to the field experience and to the technological practices, which indicate that the presence of parchment leads to prolonged maintenance of seed viability and preservation of green coffee quality with respect to the hulled beans (Selmar et al., 2008).

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# 9. General discussion

It is widely demonstrated that oxidative stress plays a crucial role in many physiological processes in plants, ranging from development to stress responses. In particular, the involvement of ROS during the physiological response of apple tree to phytoplasma invasion, named recovery (Osler et al., 1999), could be explained through two main pathways: the first, involves the production of ROS as direct antimicrobial molecules contrasting the pathogen invasion (Musetti, 2010); the second implies that ROS could act as signal molecules able to induce plant responses through the activation of hormonal pathways related to stress. The pathogenesis process was characterized by the activation of the phenylpropanoid pathway, leading to the synthesis of salicylate, while the recovery phenomen was mediated by the activation of the oxylipin pathway, a long lasting response, leading to jasmonate production (Patui et al., 2012, in press). In the case of recovered apple plants, the initial response mediated by SA, representing the first defence level was then replaced with that dependent on JA, through the activation of lipoxygenase, thus suggesting an interplay between the two hormones. A common feature of the two defence responses is ROS production, since plasma membrane NAD(P)H peroxidases were active either in symptomatic and recovered samples. Moreover, in the latter samples, NAD(P)H oxidases appear to be also stimulated. HPL, another enzyme belonging to the oxylipin pathway and involved in plant defence responses to pathogens and wound healing, was activated in both symptomatic and recovered samples. Although this enzyme does not seem specifically correlated to the recovery phenomenon, it might indicate that toxic HPL-derived products could play a role in counteracting further pathogen invasions in the canopy by altering volatile emission involved in insect vector attraction (Plotto et al., 2010; Sugio et al., 2011). Hence, a key role for the oxylipin pathway, involving signaling and/or synthesis of protective compounds, can be stated for the recovery from phytoplasma in apple tree.

The oxidative stress plays a crucial role also during germination, since many metabolic processes activated during the initial phases of embryo growth and endosperm degradation could lead to ROS production (Bailly, 2004; Muller et al., 2009; Bailly et al., 2008). In particular, germination induces the degradation of lipid storage reserves in coffee seeds, catalyzed by PLAs and lipases. The latter are key enzymes that, although still present in coffee seeds during storage, increased their activity during germination (Barros et al., 2010). While free fatty acids are readily metabolized by β-oxidation and glyoxylate cycle in germinating seeds, in commercial coffee beans these free acids could represent also a

substrate for the oxylipin pathways or free radical attack, leading to the production of lipid hydroperoxides. The hydroperoxy-octadecadienoic acids (HPODEs) were found to be accumulated in commercial coffee seeds, suggesting the presence of oxylipin pathway activity or other oxidative processes, leading to alteration of the lipid fraction. In particular, LOXs catalyze both the dioxigenation of lipid substrates (oxygenase reaction) and the secondary conversion of hydroperoxy lipids (hydroperoxidase reaction) producing short-chain alcohols and aldehydes (Patui et al., 2007). All these metabolites are detrimental for the final quality of coffee, but also for propagation material. In fact, uncoordinated mobilization of lipid fraction in germinating seeds could lead to undesired reactions, e.g. lipoperoxidation, and consequently oxidative stress that affects seed viability.

Moreover, seed viability appears of outstanding importance not only for propagation material, but also for stored commercial coffee seeds, being demostrated that longer viability of the seeds correlates to positive effect on preservation of coffee quality (Selmar et al., 2008). Selmar and co-workers also found that green coffee, stored with the parchment, remains viable longer than hulled beans. The results of this work support this concept, since parchment coffee seeds were characterized by a delay in germination and, at the same time, by a delay of lipase activity, but also by higher antioxidant capacity, with respect to seeds without parchment.

Thus, the extent of free fatty acid release, as a result of lipase activity, was dependent on the presence of the parchment and could be correlated to the lipid antioxidant capacity. These results show that the parchment acts as a barrier against gas exchange, thus limiting the oxygen diffusion, protecting the seeds either during storage or germination, similarly to what observed by Pasquini and co-workers (2012) with holm oak seed. They found that in such recalcitrant seeds, dormant-like state and consequently seed viability is prolonged when stored in plastic films that limited gaseous exchange.

In conclusion, plants, like most living organism, must face ROS production as unavoidable by-products of metabolism (Halliwell, 2006). Nevertheless, plants have evolved mechanisms not only to control, but also to exploit the production of ROS as regulative factor in many physiological processes: in this thesis, it has been evidenced how the oxidative stress, when strictly controlled and coordinated, could mediate the response to phytopathogen invasion and the lipid metabolism during the first stages of germination. Therefore, oxidative stress could be considered as a common response that the plants perform to face both biotic and abiotic stresses, respectively.

## 10. Aknowledgements

At the end of my thesis I would like to thank all those people who made this thesis possible. I would like to express my sense of gratitude to Prof. Vianello who offered his continuous advice, experience and encouragement throughout the course of this thesis.

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## 11. References


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