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Importance of Reactive Oxygen Species in Plants-Pathogens Interactions

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ABSRACT

Plant pathogens have developed various independent and well-elaborated mechanisms of penetrating and accessing plant cell contents. The production of reactive oxygen species (ROS) by the consumption of molecular oxygen during host–pathogen interactions is termed the oxidative burst. The most important ROS are singlet oxygen, the hydroxyperoxyl radical, the superoxide anion, hydrogen peroxide, the hydroxyl radical and the closely related reactive nitrogen species, nitric oxide. There are profound differences between monocots and dicots as well as in the biology of biotrophic, hemibiotrophic and necrotrophic pathogens. ROS acts synergistically in a signal amplification to drive the hypersensitive reaction (HR) and the establishment of systemic defenses. The role of ROS in successful pathogenesis, it is important to try to inhibit the cell death machinery selectively and simultaneously to monitor other defense and pathogenesis-related events. With the understanding of the molecular mechanisms underlying the localized activation of the oxidative burst following perception of pathogen avirulence signals and key downstream responses including gene activation, cell death, and long-distance signaling, novel strategies will be developed for engineering enhanced protection against pathogens by manipulation of the oxidative burst and oxidant-mediated signal pathways. In this review, it is assessed the different roles of ROS in host–pathogen interactions with special emphasis on plant pathogens.

1. Introduction

Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism. Various environmental stresses lead to excessive production of ROS causing progressive oxidative damage and ultimately cell death. Despite their destructive activity they are well described second messengers in a variety of cellular processes, including conferment of tolerance to various environmental stresses. A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids. These cytotoxic properties of ROS explain the evolution of complex arrays of nonenzymatic and enzymatic detoxification mechanisms in plants (Apel and Hirt 2004). In plants, ROS are always formed by the unavoidable leakage of electrons on to O_2 from the electron transport activities of chloroplasts, mitochondria and plasma membranes or as a by product of various metabolic pathways localized in different cellular compartments (Foyer and Harbinson 1994).

All ROS are extremely harmful to organisms at high concentrations. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of "oxidative stress". The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to death of the cells (Shah et al. 2001).

The production of reactive oxygen species (ROS) is one of the earliest cellular responses which following successful pathogen recognition. The amount of extracellular H_2O_2 is produced depends on several factors including the nature of the elicitor, the plant species, and age or developmental stages of the plant cells. Several enzymes have been implicated in apoplastic ROS generation following pathogen recognition, i.e., reduced form

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of NADPH oxidase, super oxide dismutase, oxalate oxidases, peroxidases, lipoxygenases and amine oxidases. There are profound differences between monocots and dicots as well as in the biology of biotrophic, hemibiotrophic and necrotrophic pathogens. ROS acts synergistically in a signal amplification to drive the hypersensitive reaction (HR) and the establishment of systemic defenses. The role of ROS in successful pathogenesis, it is important to try to inhibit the cell death machinery selectively and simultaneously to monitor other defense and pathogenesis-related events. Avirulent pathogens successfully recognized via the action of disease resistance (R) gene products in plant immune system. However, virulent pathogens that avoid host recognition induce only the transient, low-amplitude first phase of this response, suggesting a role for ROS in the establishment of the defenses. Elicitors of defense responses, referred to as microbe or pathogen-associated molecular patterns (PAMPs), also trigger an oxidative burst. With the understanding of the molecular mechanisms underlying the localized activation of the oxidative burst following perception of pathogen avirulence signals and key downstream responses including gene activation, cell death, and long-distance signaling, novel strategies will be developed for engineering enhanced protection against pathogens by manipulation of the oxidative burst and oxidant-mediated signal pathways (Maheshwari and Dubey 2009; Mishra et al. 2011; Srivastava and Dubey 2011).

In this review, it is presented main facts and speculations on roles of reactive oxygen species (ROS) in plant-pathogen interactions. Special attention has been attracted to the agents triggering ROS production, ROS sources and ROS involved in either resistance or compatibility and which are produced by both host and pathogen.

2. Reactive Oxygen Species (ROS)

ROS are a group of free radicals, reactive molecules, and ions that are derived from O_2. It has been estimated that about 1% of O_2 consumed by plants is diverted to produce ROS (Asada and Takahashi 1987) in various subcellular loci such as chloroplasts, mitochondria, peroxisomes. ROS are well recognized for playing a dual role as both deleterious and beneficial species depending on their concentration in plants. At high concentration ROS cause damage to biomolecules whereas at low/moderate concentration, it acts as second messenger in intracellular signaling cascades that mediate several responses in plant cells. ROS are produced in both unstressed and stressed cells at several locations in chloroplasts, mitochondria, plasma membranes, peroxisomes, apoplast, endoplasmic reticulum, and cell walls (Figure 1). ROS are always formed by the inevitable leakage of electrons on to O_2 from the electron transport activities of chloroplasts, mitochondria and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments (Sharma et al. 2010).

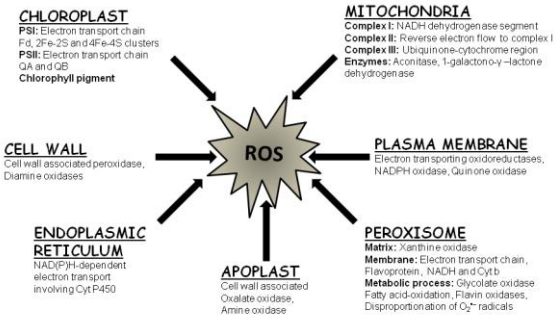


Figure 1
Sites of production of ROS in plants (Sharma et al. 2010)

The formation of cell wall appositions, papillae requiring reorganisation of the cytoskeleton and delivery of vesicles and other cell organelles such as peroxisomes, is a characteristic feature of non-host or basal resistance. Papillae consist of callose, cross-linked proteins and phenolics (Brown et al. 1998) and hydrogen peroxide is detectable by cerium chloride during their formation (Brown et al. 1998). In addition to immobilisation of the proline- and hyroxyproline-rich proteins during the apoplastic oxidative burst (Bradley et al. 1992), the induction of a number of extracellular defence proteins has long been documented before the advent of the ability to analyse global proteomes. These include the extracellular PR proteins such as chitinases, glucanases and thaumatin-like proteins (van Loon et al. 2006).

3. Functions of ROS and Oxidative Damage to Biomolecules

Production and removal of ROS must be strictly controlled in order to avoid oxidative stress. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of oxidative stress. Enhanced level of ROS can cause damage to biomolecules such as lipids, proteins and DNA (Figure 2). These reactions can alter intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein cross-linking, inhibition of protein synthesis, DNA damage, etc. ultimately resulting in cell death. ROS have been implicated as second messengers in intracellular signaling cascades that mediate several plant responses in plant cells, including stomatal closure at low/moderate concentration (Figure 2) (Kwak et al. 2003), programmed cell death (Mittler 2002), gravitropism (Joo et al. 2001), and acquisition of tolerance to both biotic and abiotic stresses (Torres et al. 2002).

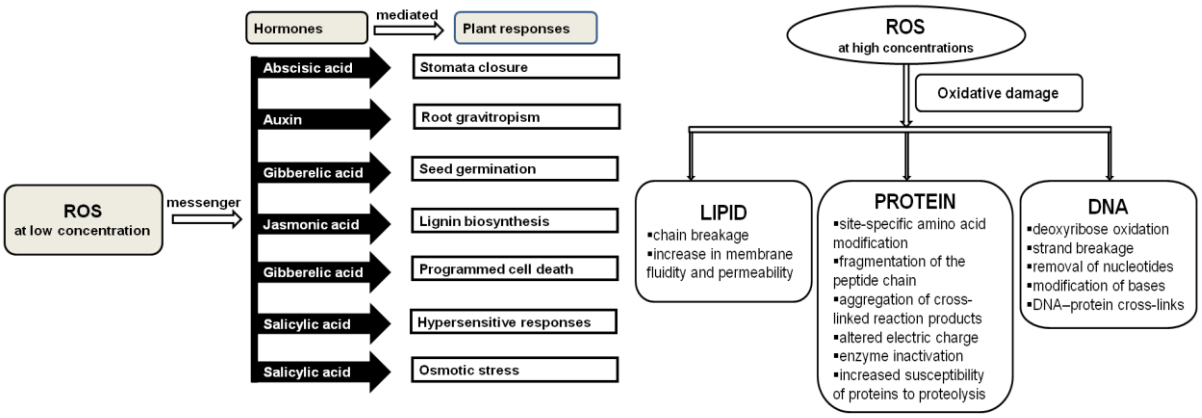


Figure 2
a. ROS as second messengers in several plant hormone responses, including stomatal closure, root gravitropism, seed germination, lignin biosynthesis, programmed cell death, hypersensitive responses, and osmotic stress
b. ROS induced oxidative damage to lipids, proteins, and DNA (Sharma et al. 2010).

4. ROS in Plant Cells

ROS are emerging as important regulators of plant development. The body of the vascular plant sporophyte (diploid life cycle stage) is derived from meristems and much of the action of development occurs where organs are formed in and around meristems. Organogenesis, the development of organs, involves an early patterning stage that roughs out boundaries where the organs will form. Within these boundaries, groups of founder cells divide and growth occurs, leading to the formation of structures containing arrays of differentiated cells. ROS are generally produced during aerobic phase of photosynthesis and photorespiration (Asada and Takahashi 1987; Kotchoni et al. 2006). Accumulation of these molecules can also be detected in peroxisomes under abiotic stress (Ramanjulu and Bartels 2002) and biotic stress (Mittler 2002). Despite being part of a normal process in the life of aerobic organism, accumulation of ROS is the source of oxidative damage and has been considered also as part of a defensive mechanism of cells. The production of ROS is recently shown to be the underlying mechanism of a series of biochemical and physiological changes that occur under environmental stress conditions, which subsequently mediate the disease resistance in plants (Gachomo and Kotchoni 2006). Barriers operating at the cell periphery to prevent invasion represent the first line of defence against pathogens that penetrate plant cells directly (Schulze-Lefert 2004). These barriers can, for example, depend on the nature and thickness of the epicuticular wax layer and cuticle or the composition and physical properties of the cell wall. Alternatively, they may occur by reinforcement of the cell wall, e.g., by deposition of callose-rich papillae and lignin at attempted penetration sites (Heitefuss 1997).

5. Role of ROS in Plant Pathogen Interactions

Involvement of ROS in signal transduction and gene expression ROS are involved in different signalling pathways for defence mechanisms, such as triggering of the HR, accumulation of phytoalexins and a number of other defence-response genes. (Mittler et al. 2004). Protein phosphorylation, changes in ion fluxes and the oxidative burst, leading to either HR or defence gene expression, or both, are important events taking place after pathogen infection (Lamb and Dixon 1997).

Doke (1983) first reported the oxidative burst, demonstrating that potato tuber tissue generated superoxide that is rapidly transformed into hydrogen peroxide following inoculation with an avirulent race of *Phytopthora infestans*. Similar H_2O_2 production is also observed during avirulent interaction between the bacteria *Pseudomonas syringae* strain DC3000 and *Arabidopsis* (Alvarez et al. 1998). Radwan et al (2010) observed higher H_2O_2 concentrations in *Vicia faba* leaves infected with Bean Yellow Mosaic Virus than those of the corresponding controls. Several enzymes have been implicated in apoplastic ROS production following successful pathogen recognition. The use of inhibitors pointed to plasma membrane NADPH oxidases and cell wall peroxidases as the two most likely biochemical sources (Grant et al. 2000). The expression of these enzymes is induced following recognition of bacterial and fungal pathogens (Sasaki et al. 2004). Although the primary oxidative burst following pathogen recognition occurs in the apoplast, ROS can be produced in other cellular compartments like mitochondria and chloroplast. Abdollahi and Ghahremani (2011) studied the role of chloroplasts in the interaction between *Erwinia amylovora* and host plants by using uracil as chloroplast ETC inhibitor. Uracil presence significantly reduced ROS

generation during pathogen-host interaction, and ROS generation corresponded with the appearance of necrosis in all cultivars.

The actual toxicity of ROS in a given plant–pathogen interaction will depend on the sensitivity of the pathogen to the concentration of ROS present (Levine et al. 1994). The amount of extracellular H_2O_2 produced depends on several factors including the nature of the elicitor, the plant species, and age or developmental stages of the plant cells (Legendre et al. 1993; Nurnberger et al. 1994). Micromolar concentrations of H_2O_2 inhibited spore germination of a number of fungal pathogens in vitro (Peng and Kuc 1992). Thus, a concentration of 0,1 mM H_2O_2 completely inhibited the growth of cultured bacteria *Pectobacterium carotovorum* subsp. *carotovorum* and resulted in >95% inhibition of *Phytophthora infestans* growth (Wu et al. 1995).

The most abundant information that links ROS production and innate resistance to diseases corresponds to complete (vertical, monogenic) resistance which prevents disease very effectively but only in specific host-parasite combinations. In contrast, partial (horizontal, general, quantitative, polygenic) resistance is unspecific towards various pathogen races but protects plants to lesser extent than successful complete resistance. Completely resistant cultivars prompt pathogens to evolve virulent races which break the resistance down. Partial resistance is better in this regard as it is not such a strong elective factor and so is more durable (Desikan et al. 1996). For example, such interactions of *Uromyces vignae* with pea or *Erisiphe cichoraceum* with cowpea are accompanied by increased H_2O_2 and O_2^- production (Asada 1999).

Based on studies of innate immunity in Arabidopsis, suggests that pathogens or PAMPs are recognised by receptors which trigger an ion (calcium) channel, leading to increases in cytosolic Ca_2^+ and subsequent nitric oxide (NO) generation (Ali et al. 2007). NO generation, together with other required factors such as an avirulent pathogen and an oxidative burst, could lead to the HR and potentially, diffusion of NO to neighbouring cells could act as a signal that thereby activates further calcium channels. Activation of the oxidative burst is governed by phosphorylation/dephosphorylation (Lamb and Dixon 1997).

Similarly, salicylic acid and the hormone jasmonic acid seem to either synergize or antagonize in their signaling functions at different concentrations. Synergy, in this case, drives ROS production and cell death (Mur et al., 2006). There is evidence for the interaction of ROS and ethylene. Ethylene is known to induce programmed cell death and fruit or flower senescence, and there is also evidence for the accumulation of H_2O_2 in response to ethylene in tomatoes (de Jong et al. 2002). Additional experiments also highlighted the interplay between ROS and ethylene signalling in *Arabidopsis* resistance against Cauliflower Mosaic Virus (Love et al. 2005) .

Antioxidative defense system in plants

Plants possess complex antioxidative defense system comprising of non-enzymatic and enzymatic components to scavenge ROS. In plant cells, specific ROS producing and scavenging systems are found in different organelles such as chloroplasts, mitochondria, and peroxisomes. ROS-scavenging pathways from different cellular compartments are coordinated (Pang and Wang 2008). Under normal conditions, potentially toxic oxygen metabolites are generated at a low level and there is an appropriate balance between production and quenching of ROS. The balance between production and quenching of ROS may be perturbed by a number of adverse environmental factors, giving rise to rapid increases in intracellular ROS levels (Noctor et al. 2002; Sharma et al. 2010).

a. Non-enzymatic components of antioxidative defense system

Non-enzymic components of the antioxidative defense system include the major cellular redox buffers ascorbate (AsA) and glutathione (-glutamyl-cysteinyl-glycine, GSH) as well as tocopherol, carotenoids and phenolic compounds(flavonoids, tannins, hydroxycinnamate esters, and lignin). They interact with numerous cellular components and in addition to crucial roles in defense and as enzyme cofactors, these antioxidants influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and cell death. Mutants with decreased non-enzymic antioxidant contents have been shown to be hypersensitive to stress (Gao and Zhang 2008; Semchuk et al. 2009).

b. Enzymatic components

The enzymatic components of the antioxidative defense system comprise of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH) cycle ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer 1998). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress. Several enzymes have been implicated in apoplastic ROS production following successful pathogen recognition. The use of inhibitors pointed to plasma membrane NADPH oxidases and cell wall peroxidases as the two most likely biochemical sources (Grant et al. 2000). The expression of these enzymes is induced following recognition of bacterial and fungal pathogens (Sasaki et al. 2004).

Antioxidant enzymes known to be present in Pseudomonas include superoxide dismutase (SOD), an enzyme capable of producing hydrogen peroxide from the superoxide radical. Three types of SOD exist in bacteria, distinguished by their metal cofactors: Mn/Fe, Cu-Zn and Ni (Kim et al. 1999). Protection from hydrogen peroxide is provided by the hydrogen peroxide-degrading

enzyme catalase and also peroxidases (Albert et al. 1986). Genome sequence analyses indicate that *P. syringae* pv. *tomato* DC3000 possesses three SODs (Mn-SOD, Fe-SOD and Cu-Zn-SOD), three catalases and six peroxidases (Buell et al. 2003). While ROS-degrading enzymes are common in pathogen genomes and may act as virulence factors (Soto et al. 2006), their importance for bacteria is not entirely understood, and some studies have provided conflicting evidence about their role in ROS tolerance. Cu-Zn SODs are not only present in plant pathogenic strains of *P. syringae* but also predicted to be present in a wide range of plant pathogenic and plant symbiotic bacteria, including *Agrobacterium* spp., *Rhizobium* spp., *Xanthomonas* spp., *Ralstonia solanacearum*, *Burkholderia* spp. and *Pectobacterium atrosepticum*, suggesting that these enzymes play a broadly conserved and important role in plant pathogenesis (Finn et al. 2010). Nevertheless, other types of SOD have been shown to be important in some plant–pathogen interactions, as the soft-rot pathogen *Dickeya dadantii* has been shown to require Mn-SOD activity for the successful maceration of *Saintpaulia ionantha* leaves, although interestingly the Mn-SOD mutant retained the ability to macerate potato tubers (Santos et al. 2001).

In tobacco, the reduction of CAT and APX activities resulted in plants hyper-responsive to pathogens (Mittler *et al*., 1999). Significant increase in the activities of POD and CAT was observed in leaves of flax lines infected with powdery mildew (Ashry and Mohamed 2012). Increase in POD activity was much pronounced in tolerant lines than susceptible lines. Enhanced activities of POD, CAT, APX, and SOD were observed in *Vicia faba* leaves infected with Bean Yellow Mosaic Virus (Radwan et al. 2010).

ROS phytotoxicity in plants

The hypersensitive reaction (HR) is a rapid host response occurring in a host cell, which is infected by a pathogen (Lam 2004). The cells die shortly after penetration often together with some of the surrounding cells (Greenberg 1997). The best-known ROS-dependent anti-infection phytotoxic effect is the HR. It is a rapid death of invaded plant cells resulting in the parasite death or cessation of its development. The HR occurs in order to restrict pathogen growth and is highly effective against biotrophic pathogens, since, with the death of host cells, the nutrient supply is removed (Greenberg and Yao 2004).

The HR is often not effective against necrotrophic pathogens because these usually kill host cells to feed on them (Mayer et al. 2001). Thus, for true necrotrophic pathogens, such as *Botrytis cinerea*, it has been suggested that plant cell death is beneficial for infection, leading to enhanced colonisation (Greenberg and Yao 2004). In addition, there is a group of pathogens, often considered to be necrotrophic, which are in fact inhibited to some extent by HR, e.g., *Pyrenophora teres* (Jorgensen et al. 1998).

The HR is a type of active PCD (Lam 2004), which is often characterised by discrete cellular lesions. H_2O_2 is accumulating throughout the tissue in which fungal sporulation occurs by an oxidative burst (Baker and Orlandi 1995). The process of HR may involve several steps including chromatin condensation, DNA cleavage and membrane blebbing, eventually leading to membrane disruption and release of cell contents (Li et al. 2006). Elucidation of the causal relation between ROS and HR is further complicated by the fact that, for example, plant hormones such as SA, JA, ET and abscisic acid also influence the elicitation and expression of HR (Torres et al. 2005).

There are also reports where elicitors and pathogens have been shown to trigger a strong oxidative burst without causing an HR but activate other defence mechanisms involved with the oxidative burst (Glazner et al. 1996). The researchers showed that ROS accumulation in tobacco leaves and cultured cells in response to an incompatible strain of *Pseudomonas syringae* pv. *syringae* was not sufficient to cause HR.

6. Mechanisms of ROS Production in Response to Pathogens

ROS play a central role in plant pathogen defense. Several enzymes have been implicated in apoplastic ROS production following successful pathogen recognition. The use of inhibitors pointed to plasma membrane NADPH oxidases (inhibited by diphenylene iodonium (DPI) but not by cyanide or azide and cell wall peroxidases (inhibited by cyanide or azide but not by DPI; Grant et al. 2000a; Bolwell et al. 2002) as the two most likely biochemical sources.

Peroxidases form a complex family of proteins that catalyze the oxidoreduction of various substrates using H_2O_2. In particular, pH-dependent peroxidases in the cell wall can also be a source of apoplastic H_2O_2 in the presence of a reductant released from responding cells (Wojtaszek 1997; Bolwell et al. 1998). The expression of these enzymes is induced following recognition of bacterial and fungal pathogens (Chittoor et al., 1997; Sasaki et al., 2004). Various ROS-scavenging systems, including ascorbate peroxidases, glutathione, superoxide dismutases, and catalases, maintain ROS homeostasis in different compartments of the plant cell (Mittler et al. 2004). These enzymes could restrict the ROS-dependent damage or finely tune ROS-dependent signal transduction. Differential regulation of these enzymes, in part mediated by SA, may contribute to increases in ROS and activation of defenses following infection (Dorey et al., 1998; Mittler et al. 1999).

ROS production has been associated with the formation of defensive barriers against powdery mildew in barley (Huckelhoven and Kogel 2003). ROS produced in the barley/powdery mildew interaction were observed in vesicles inside the cell, suggesting that the polarized

delivery of ROS, among other factors, might contribute to inhibition of pathogen growth (Collins et al. 2003).

7. Role of ROS Following Infection

Following the recognition of an oxidative burst in the plant defence response against pathogen attack, the earliest studies concentrated upon the biochemistry of production of ROS including hydrogen peroxide and superoxide (Bolwell and Wojtaszek 1997; Lamb and Dixon 1997). NADPH oxidases have been implicated in biotic interactions in a number of plants especially *Arabidopsis* and Solanaceous species (Torres and Dangl 2005) . They were first identified by the susceptibility of the ROS production in plants to inhibition by diphenylene iodonium (DPI). *Arabidopsis thaliana* suspension-cultured cells also show an azide-sensitive but DPI-insensitive apoplastic oxidative burst that generates H2O2 in response to a *Fusarium oxysporum* cell wall preparation (Bindschedler et al. 2006; Bolwell et al. 2002).

Though the involvement of an NADPH oxidase has been predominant in most cases (Bolwell et al. 1998; Grant et al. 2000b; Torres and Dangl 2005), both NADPH oxidases and cell wall peroxidases might mediate ROS production in response to the same pathogen (Grant et al. 2000a). A more detailed temporal resolution of the activity of each system may reveal that the pools of ROS produced by each mechanism do not functionally overlap. For example, differential effects of DPI on ROS accumulation during the HR- and MAMP-mediated basal defense responses were reported, with the latter being considerably less attenuated by DPI (Soylu et al. 2005). These results suggest that alternative mechanisms might be activated to produce ROS during some basal defense responses, while NADPH oxidases might have later effects following R-mediated pathogen recognition.

ROS, in association with SA, were proposed to mediate the establishment of systemic defenses (systemic acquired resistance; SAR, Durrant and Dong 2004). The rapidity of ROS production and the potential for H_2O_2 to freely diffuse across membranes suggested that ROS could function as an intercellular or intracellular second messenger (Levine et al. 1994; Lamb and Dixon 1997). ROS metabolism could also affect the function of NPR1, a crucial mediator of these systemic responses, by controlling NPR1 redox state (Mou et al., 2003). However, although H_2O_2 may mediate the accumulation of defense markers beyond the initial infection site, inhibitor studies indicate that it is unlikely that it is itself the translocated signal that mediates SAR (Dorey et al. 1999; Costet et al. 2002), and genetic proof will be needed to clearly establish the role, if any, of ROS in SAR. Interestingly, there is also evidence that NADPH oxidase mediates the systemic production of ROS in response to successful viral infection in Arabidopsis, although the functional relevance of this remains unclear (Love et al., 2005).

Elicitors of defense responses, often referred to as microbe-associated molecular patterns (MAMPs), also trigger an oxidative burst. Initial characterization of the oxidative burst left unclear whether ROS acted as executioners of pathogen, host cells (in the form of the familiar HR), or both, or, alternatively, as signaling molecules that were not directly involved in the mechanisms that actually stopped pathogen growth. In the plant cell, ROS can directly cause strengthening of host cell walls via cross-linking of glycoproteins (Bradley et al. 1992; Lamb and Dixon 1997), or lipid peroxidation and membrane damage (Montillet et al. 2005). However, it is also evident that ROS are important signals mediating defense gene activation (Levine et al. 1994).

Although ROS usually correlates with successful disease resistance responses, some pathogens may induce production of ROS to their own advantage. For example, necrotrophs appear to stimulate ROS production in the infected tissue to induce cell death that facilitates subsequent infection (Govrin and Levine 2000). The fungal necrotroph Botrytis triggers significant changes in the peroxisomal antioxidant system, leading to a collapse of the protective mechanism at advanced stages of infection (Kuzniak and Sklodowska 2005). Interference with the chlorophyll degradation pathway also results in overaccumulation of ROS and an increase in susceptibility to some necrotrophic pathogens (Kariola et al. 2005). In addition, there are also reports of ROS being produced, together with increased levels of ROS detoxification enzymes, during compatible interactions involving virus (Allan et al. 2001; Clarke et al. 2002). Some proteins of the Rac family also appear to function in pathogen susceptibility (Schultheiss et al. 2003). Thus, ROS is produced as part of a complex network of signals that respond to pathogen attack and mediate multiple responses, sometimes with opposite effects, in different contexts or in response to different pathogens.

8. ROS in Successful Pathogenesis

Plants have evolved a complex regulatory network to mediate biotic and abiotic stress responses based on ROS synthesis, scavenging and signaling. Transient production of ROS is detected in the early events of plant-pathogen interactions and plays an important signaling role in pathogenesis signal transduction regulators (Nanda et al. 2010).

Cell death plays a different role in plant response to biotrophs and necrotrophs (van Doorn et al. 2011). HR cell death contributes to resistance to biotrophic pathogens by confining the pathogen and limiting its growth (Jones and Dangl 2006). The role of ROS in successful pathogenesis are based solely on correlative data and come from rather few pathosystems. *B. cinerea* is most often used as a representative necrotrophic pathogen (Shetty et al. 2007).

Biotrophic pathogens obtain their nutrition from living host cells (Oliver and Ipcho 2004), and H_2O_2 has

been reported as an effective factor in stopping growth of biotrophic pathogens such as *B. graminis* f. sp. *hordei* (Trujillo et al. 2006). Biotrophic pathogens may suppress the host defence responses during infection (Ferreira et al. 2007). For example, the fungal metabolite mannitol, which can suppress ROS-related defence mechanisms by scavenging ROS, was found in apoplastic fluids of *Vicia faba* leaves infected with *Uromyces fabae* (Link et al. 2005).

A correlation between pathogen growth at the late stages of their life-cycle and large quantities of H_2O_2 has also been reported in such host-pathogen systems. Shetty et al. (2003) observed a similar correlation in wheat infected by the hemibiotrophic pathogen *S. tritici*. During the biotrophic phase of the interaction, H_2O_2 accumulation occurred as a defence response only in an incompatible interaction.

Sensing and stopping pathogen penetration is among the primary detecting mechanisms associated with disease resistance in plants. The detecting machinery of pathogen infection by the host cells include cross-linking of preexisting or else induced cell-wall proteins and phenolic compounds (Davletova *et al* 2005), formation of calcium-pectate gels (Kieffer et al. 2000), accumulation of glycoproteins (Mazau and Esquerre-Tugaye 1986), silica (Aist and Bushnell 1991), deposition of callose-containing pappille (Aist and Bushnell 1991) and the generation of ROS (Thordal-Christensen et al. 1997). Generally, production of these compounds occurs simultaneously to mount a programmed and efficient infection arrest in plants (Perumalla and Hearth 1991).

Several microbial toxins may work as elicitors inducing resistance preceded by stimulation of ROS production in treated plants. Picolinic acid, the toxin of *Magnaporthe* and *Fusarium* fungi, elicits the burst of H_2O_2 production and cell death in leaves and suspension culture of rice. Leaf pretreatment with the toxin diminishes severity of subsequent inoculation with blast (Petit et al. 2001). Another blast toxin, tenuazonic acid also causes leaf necrosis. Adding the toxin to spore inoculum applied to leaves of susceptible rice cultivar increased the percentage of incompatible-type necrotic spots and decreased that of compatible-type lesions, which also acquired brown margin. In other words, the disease symptoms shifted from compatible to incompatible. In disease-controlling doses, the compound was not toxic to spores but increased the fungitoxicity of diffusates of treated leaves in ROS-dependent manner (Polidoros et al. 2001).

Several pathovars of *P. syringae* produce a phytotoxin known as coronatine, which is known to be necessary for virulence of this pathogen (Bender et al. 1987; Uppalapati et al. 2008). Coronatine has a number of functions in planta, including acting as a mimic of the plant hormone methyl jasmonate to antagonistically suppress salicylate-based defences (Zhao et al. 2003). It is also known to be involved in symptom development, causing a chlorotic halo around the infection site, owing to a loss of chlorophyll a and b contents (Ishiga et al. 2009). Loss of chlorophyll is correlated with a large reduction in the efficiency of photosytem II, owing to a coronatine-induced downregulation of genes involved in chlorophyll synthesis, photosystem proteins, oxygen evolving complex proteins and the Calvin cycle, as well asthe induction of chlorophyllase (Ishiga et al. 2008). It has recently been found that this loss of photosynthetic ability is associated with the light-dependent generation of ROS and downregulation of thylakoid Cu-Zn SOD activity. This ROS generation appears to be necessary for the development of the necrotic lesions that characterize the bacterial speck disease caused by this pathogen (Ishiga et al., 2008).

Different members of gene families involved in the protective mechanism against pathogen attacks were upregulated in plants under exogenous application of ROS (Rizhsky et al 2004). Mellersh et al (2002) demonstrated that localized generation of H_2O_2 is one of the earliest cytologically detectable defence responses to penetration of plant cell walls by various fungal pathogens. Rapid generation of H_2O_2 in response to cell wall penetration is one of the most important determinants of pathogen penetration failure in invading epidermal cells (Mellersh et al 2002).

Enzymatic removal of H_2O_2 resulted in increased penetration success of fungi in the host plant cells (Mellersh et al 2002). Although the chemical nature and reactivity of ROS prove them to be potentially harmful to cells, plants use them as secondary messengers in signal transduction cascades regulating diverse processes such as mitosis, tropisms, cell death and defence mechanisms (Pavet et al 2005). Exogenous application of H_2O_2 was found to be essential to activate different pathogenesis-related proteins and to provide adequate protection against the pathogenic fungus *Diplocarpon rosae* causing black spot disease of rose leaves (Gachomo and Kotchoni 2006). Knock-out (KO) plants deficient in ROS-scavenging proteins are of particular interest in elucidating role of ROS in disease defence systems. They have been used to study implication of high ROS content in plants response to pathogens infections. These KO-plants maintain a high steady-state level of H_2O_2 in cells and activate ROS defence mechanisms when grown under control conditions (Pnueli et al 2003*;* Davletova et al 2005). These mutant plants provide an ideal experimental system to study plant responses to ROS accumulation and the effect of ROS mediating the activation of environmental stress-related proteins (Davletova et al 2005; Kotchoni et al 2006). On the other hand, transgenic plants expressing H_2O_2-generating enzymes have been reported to display increased protection against bacterial and fungal pathogens (Wu et al 1995; Schweizer et al 1999). Treatments with elicitors, such as CFs of *Pectobacterium carotovorum* subsp. *carotovorum* and chitosan, also provoked cell death in *P. patens* tissues (Lawton and Saidasan 2009). Harpin

proteins from *Pectobacterium* sp. (Wei et al. 1992; Kariola et al. 2003), *Xanthomonas axonopodis* (Kim et al. 2004) or *Pseudomonas syringae* (Alfano et al. 1996) elicit HR in flowering plants.

H_2O_2 most likely acts within a pathway involving transcription/translation and the expression of wall-associated responses such as the accumulation of fungal inhibiting compounds (Aist and Brushnell 1991). Recent studies suggest that cross-talk between salicylic acid (SA), jasmonic acid (JA), and ethylene-dependent signalling pathways regulates plant responses to both bioticand abiotic stress factors. Although sublethal H_2O_2 concentrations induce expression of defence genes, complete induction of defence genes and cell death requires additional signalling molecules such as SA at the whole-plant level (Chamnongpol et al 1998; Rao and Davis 1999). Among several molecules proposed to act downstream of ROS, SA, JA, and ethylene are considered to be the major regulators of plant defence responses (Veronese et al 2006).

9. ROS in Molecular Plant–Pathogen Interactions

The plasma membranes of plant cells contain extracellular surface pattern-recognition receptors (PRRs), which are able to detect signals from invading pathogens (MAMPs) and elicit basal resistance. This leads to several early responses such as MAP kinase signalling cascades, transcriptional induction of defence genes, rapid microbursts of ROS and callose deposition to strengthen the cell wall at sites of infection as a result of complex cellular remodelling (Chisholm et al. 2006; Nurnberger et al. 2004). A well-studied example of such basal resistance is the response to the bacterial protein (Gomez-Gomez and Boller 2002).

The HR is dependent on the activation of *R* genes and numerous examples have been characterised in several different plant species against important pathogens (Hammond-Kosack and Parker 2003). An abundant class of plant R genes encode intracellular nucleotide-binding/leucine-rich repeat (NB-LRR) proteins with variable N-terminal domains (Meyers et al. 2005). Plant NB-LRR proteins have evolved to recognise *avr* products of pathogens (He et al. 2007).

The type three secretion system allows the bacteria to deliver effector proteins (TSSE), some of which delay or inhibit the plant's defence responses, including the production of ROS (Grant et al., 2006). However, it is important to note that the production of ROS also occurs in compatible reactions between plant and pathogen, in which TTSE are successfully deployed and disease develops (Kim et al., 1999), albeit to a lesser extent than during an incompatible, nonhost reaction.

An additional and relatively unexplored role for ROS tolerance in plant–pathogen interactions is suggested by studies of bacterial cell death mechanisms in response to bactericidal antibiotics. Kohanski et al. (2007) have shown that bactericidal antibiotics belonging to the quinolone, aminoglycoside and b-lactam family induce production of hydroxyl radicals as the end product of an oxidative cell death pathway in bacteria.

An important factor in a bacterial pathogen's ability to with stand the oxidative burst is its coating of extracellular polysaccharides (EPS), which act to protect the bacterium against oxidative stress. Examples of EPS found in *Pseudomonas* species include alginate and levan (Chang et al., 2007). The EPS of *P. syringae* and *P. aeruginosa* are known to be upregulated by exposure to ROS (Keith and Bender, 1999). Keith et al. (2003) studied the expression of the algD gene, involved in alginate production, in planta, and found evidence that this gene is upregulated in response to ROS produced by the plant and that this induction of alginate production occurs in both compatible and incompatible plant–pathogen interactions (Keith et al., 2003). The activities of antioxidant enzymes and EPS in bacterial responses to ROS-stress are illustrated in Figure. 4.

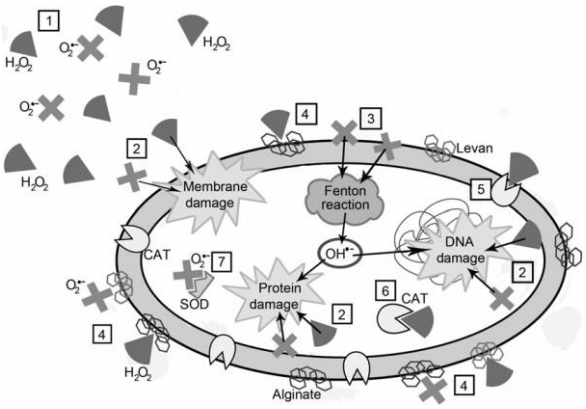
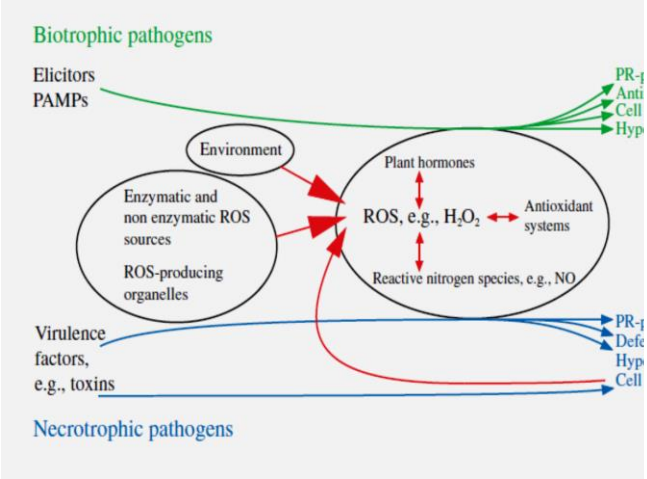


Figure 3

Summary of interactions between bacterial ROS tolerance mechanisms and host ROS-based defences. ROS, including H_2O_2 and $O_2^{\cdot-}$, are produced at the plasma membrane of plant cells following detection of a pathogen (1). These ROS have antimicrobial activity, causing damage to bacterial protein, DNA and membranes (2). $O_2^{\cdot-}$ can also participate in Fenton reactions, leading to the generation of highly reactive $OH^{\cdot-}$radicals, causing further damage (3). Bacterial defences include EPS (4) and periplasmic catalase (5), which can neutralize ROS prior to their entering the bacterial cell. Once inside the cell, ROS may be disarmed by cytoplasmic catalase (6) and SOD (7) (Fones and Preston 2012)

Systemic acquired resistance (SAR) is associated with the increase in expression levels of several well characterised defence-related or pathogenesis-related (PR) genes (Durrant and Dong 2004). Reactive oxygen species appear to be involved in the establishment of systemic defences in conjunction with salicylic acid (Kanzaki et al. 2003). A moderate concentration of ROS activates the cellular defence response (Levine et al

1994). Tobacco plants inoculated with the Tobacco Mosaic Virus (TMV) developed SAR that was mediated by a burst of ROS (Lamb and Dixon 1997). At all levels, successful pathogens can potentially suppress ROS production as a component of basal resistance, HR or SAR. ROS are involved in different signaling pathways for defense mechanisms, such as triggering of the HR, accumulation of phytoalexins and a number of other defense-response genes (Shetty et al. 2008) (Figure 3).



Figue 3

Adopted sources and functions of ROS in host–pathogen interactions of biotrophic and necrotrophic pathogens (Shetty et al. 2008)

Response of plants to pathogens depends on the regulatory gene expression at genomic level through complex genetic controls influenced by redox regulation (Pavet et al. 2005). Microarray technology has provided new insight into the transcriptomes involved in mechanisms of environmental stress tolerance in higher plants. Several robust screening methods such as DNA microarry analysis, mass spectrometry-based proteomics and forward/reverse genetics (Tyres and Mann 2003) are being now used to identify stress-inducible genes at genomic level. Genomics-based approaches, and bioinformatic tools are now available and being used to identify specific set of genes that are up-regulated by ROS. For example, Scheideler et al (2002) have monitored, simultaneously global changes in the *A. thaliana* transcriptome after infecting the plant with the incompatible bacteria pathogen *Pseudomonas syringae* pv. tomato by using cDNA arrays comprising 13000 unique expressed tags.

Transfer of genes responsible for ROS hyper-production may render plant resistant. A gene from *Aspergillus niger* that encodes glucose oxidase (yielding hydrogen peroxide from the oxidation of glucose) was inserted into potato plants. As a result, leaves and tubers produced high amounts of H_2O_2 constitutively and acquired resistance to the bacterium *Pectobacterium carotovorum* subsp. *carotovorum* as well as fungi *P. infestans* and *Verticillium dahliae*. This acquired resistance does not occur if the pathogen inoculum contains catalase indicative the phenomenon really depends on enhanced H_2O_2 level (45).

While the Rboh proteins are required for ROS production following successful pathogen recognition, these ROS may serve diverse signaling functions in disease resistance. NADPH oxidase-Rboh function in the pathogen-induced oxidative burst came from the analysis of rboh mutants and antisense lines. Down-regulation or elimination of Rboh leads to elimination of extracellular peroxide formation. Yet, this lack of ROS has variable effects on pathogen growth and HR. For example, a double mutant of the Arabidopsis atrbohD and atrbohF genes displays reduced HR in response to avirulent bacteria For example, *AtrbohD* and *AtrbohF* genes were identified as required for the production of a full oxidative burst in response to avirulent strains of the bacterial and oomycete pathogens *Pseudomonas syringae* and *Hyaloperonospora parasitica*, respectively (Torres et al. 2002) .

Different plant Rac proteins appear to act as either positive or negative regulators of ROS production. For example, Osrac1 is a positive regulator of ROS production and cell death (Ono et al., 2001), whereas Ntrac5 acts as a negative regulator of ROS production via NtrbohD (Morel et al., 2004). These analyses suggest that combinations of Rac isoforms with specific Rboh isoforms may mediate differential regulatory outcomes and could explain the differential functions of NADPH oxidases in regulation of defense and cell death.

10. Discussion

The oxidative burst in plant-pathogen interactions has advanced considerably since the first reports, there are still several unanswered questions. The rapid production of ROS in the apoplast in response to pathogens has been proposed to orchestrate the establishment of different defensive barriers against pathogens (Torres et al. 2006). Complexity of plant responses to multiple stresses has shown a need to develop new research approaches to elucidate the overwhelming benefit of ROS in plant defense mechanisms. With the advance of biotechnology, ribosome inactivating proteins can now be used as a potential tool to engineer plants resistant to various stresses (Nielson and Boston 2001).

There are profound differences between monocots and dicots as well as in the biology of biotrophic, hemibiotrophic and necrotrophic pathogens. Caution should therefore be exercised before stating that processes occur in a similar way in totally different systems.

In a genetic approach, using mutants, gene silencing, gene knock-outs and/or over-expression, careful physiological and biochemical characterisation of different host–pathogen interactions and defence responses activated should be carried out followed by studies of the

role of proteins encoded by ROS genes in the different systems. This approach provides insight into their precise function in defence, cell death, and/or pathogen development, through determination of their sub-cellular localisation and biochemical function.

The notion of ROS accumulation mediating the up-regulation of specific set of stress inducible genes is a positive aspect of plant defence systems, which needs to be evaluated in various agricultural important crops and the ROS network pathways may be adopted as a highly beneficial pre-requisite for disease resistance in plants.

It is also important to study the interplay between ROS and SA/NO, in order to gain further insights into the regulation of resistance, as these are important defence response regulators that interact with ROS signalling in response to pathogens (Mur et al. 2006). Thus, ROS may be part of many signalling pathways and provide a crucial link in the cross-talk to different responses (Apel and Hirt 2004). The flux of information between different cell compartments needs to be elucidated to further understand the regulatory capabilities of ROS. Previously, genetic engineering for improved disease resistance has mainly targeted genes involved in the recognition of the pathogen or in the over-expression of defence molecules like phytoalexins (Jalali et al. 2006).

It is clear that ROS play a key role in plant–pathogen interactions; they are used by plants as a weapon against pathogens via direct toxicity and are important effectors in bacterial cell death mechanisms. Successful pathogens must therefore be able to tolerate this threat. But plants also use ROS in signalling, which bacteria may be able to manipulate for their own ends or to downregulate to avoid further defence responses.

Further work is needed to fully illuminate a number of the areas covered in this review. There is yet to be a full understanding of the consequences of the changes observed in infected plants in this complex and dynamic process.

ROS-generating systems of plants and pathogens may be targets for stimulation by resistance inducers or fungicides. Other directions of chemical attack on microbes are their antioxidants and ROS-depended regulatory systems. Genes involved in the oxidative burst may be used to create resistant transgenic plants. For diseases where ROS favor pathogenicity, artificial induction of antioxidant potential may be used to weaken the disease. In addition to manipulations on hosts and parasites, the usage of the third power, namely, ROS-depended biocontrol microbes and their products, seems to offer another concept for the application of ROS to agriculture.

Study of formation and fate of ROS using advanced analytical techniques will help in developing broader view of the role of ROS in plants. Future progress in genomics, metabolomics, and proteomics will help in clear understanding of biochemical networks involved in cellular responses to oxidative stress. Improved understanding of these will be helpful in producing plants with in-built capacity of enhanced levels of tolerance to ROS using biotechnological approach.

11. References

Abdollahi H, Ghahremani Z (2011). The role of chloroplasts in the interaction between *Erwinia amylovora* and host plants. *Acta Horticulturae* 896: 215-221.

Aist J R, Brushnell WR (1991). Invasion of plants by powdery mildew fungi, and cellular mechanisms of resistance; in: *The fungal spore and disease interaction in plants and animals* (eds) G T Cole and H C Hoch (New York: Plenum Press) pp 321–345

Albert FG, Benet LW, Anderson AJ (1986). Peroxidase associated with the root surface of *Phaseols vulgaris. Canadian Journal of Botany* 64: 573–578.

Alfano JR, Bauer DW, Milos TM Collmer A (1996). A. Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally nonpolar hrpZ deletion mutations, truncated HrpZ fragments, and hrmA mutations. *Molecular Microbiology* 19: 715–728.

Ali R, Ma W, Lemtiri-Chlieh, F, Tsaltas, D, Leng Q, von Bodman S (2007). Death don't have no mercy and neither does calcium: Arabidopsis Cyclic Nucleotide Gated Channel 2 and innate immunity. *The Plant Cell* 19: 1081–1095.

Allan AC, Lapidot M, Culver JN, Fluhr R (2001). An early tobacco mosaic virus-induced oxidative burst in tobacco indicates extracellular perception of the virus coat protein. *Plant Physiology* 126: 97–108

Asada K (1999). The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology Plant Molecular Biology* 50:601–39

Asada K, Takahashi M (1987). "Production and scavenging of active oxygen in photosynthesis," in *Photoinhibition*: *Topics of Photosynthesis*, DJ Kyle, CB Osmond, CJ Arntzen, Eds., pp. 227-287, Elsevier, Amsterdam, 9th edition,

Baker CJ, Orlandi EW (1995). Active oxygen in plant pathogenesis. *Annual Review of Phytopathology* 33: 299– 321.

Bender CL, Stone HE, Sims JJ, Cooksey DA (1987). Reduced pathogen fitness of Pseudomonas syringae pv. tomato Tn5 mutants defective in coronatine production. *Physiological and Molecular Plant Pathology* 30: 272–283.

Bindschedler L, Dewdney V, Blee J, Stone KA, Asai JM, Plotnikov T, (2006). Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *The Plant Journal* 47: 851–863.

Bolwell GP, Wojtaszek P (1997). Mechanisms for the generation of reactive oxygen species in plant defence – a broad perspective. *Physiological and Molecular Plant Pathology* 51: 347 – 366

Bolwell GP, Bindschedler LV, Blee KA, Butt VS, Davies DR, Gardner SL, Gerrish C, Minibayeva F (2002). The apoplastic oxidative burst in elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257: 85–88.

Bolwell GP, Davies DR, Gerrish C, Auh CK, Murphy TM (1998). Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. *Plant Physiology* 116: 1379–1385.

Borsani O, Valpuesta V, Botella MA (2003). Developing salt tolerance plants in a new century: a molecular biology approach. *Plant Cell Tissue Organ Culture* 73: 101–115

Bradley D, Kjellbom P, Lamb C (1992). Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Plant Cell* 70: 21–30.

Britto D T, Kronzucker HJ (2001). Can unidirectional influx be measured in higher plants? A mathematical approach using parameters from efflux analysis; *New Phytology* 150 37–47

Brown I, Trethowan J, Kerry M, Mansfield J, Bolwell GP (1998). Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed duringthe interaction bet ween non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. *Plant Journal* 15 333–343.