Oxidative processes involved in soft rots caused by *Botrytis cinerea*

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Botrytis cinerea causes grey mould disease and associated soft rots in a wide range of horticultural crops world-wide. The fungus is a necrotroph, whereby it invades plant tissues by killing cells in advance of mycelial penetration and is subsequently able to grow on dead tissues. In contrast, biotrophs, such as rusts and mildew, are only able to grow in living plant cells. There are therefore some fundamental differences in the type and speed of some of the molecular events associated with the infection process between biotrophs and necrotrophs. Previously, emphasis has been placed on investigating the role of cell wall degrading enzymes and toxin production during infection by *B. cinerea*. As partners in an EU-funded project named 'Oxidative attack by necrotrophic pathogens-new approaches for an innovative and non-biocidal control of plant diseases' (AOS-PLANT), we have investigated the oxidative burst during infection with an emphasis on quantifying redox-related changes through electron paramagnetic resonance (EPR) spectroscopy[1], quantification of compounds influencing the redox state of cells, and chemical markers for consequential lipid peroxidation.

Increasing evidence suggests that oxidative processes involving highly reactive free radicals (chemical species with unpaired electrons), metal ion species and toxic products of the peroxidation of lipids present in cell membranes are involved in the disease processes in plants in a manner analogous to processes in animal cells. We have studied some of these oxidative processes in fruits of sweet pepper (*Capsicum annuum*) and leaves of French bean (*Phaseolus vulgaris*) and *Arabidopsis thaliania* during necrotrophic infection by *B. cinerea*. Comparison of cellular processes using tissues from these three plant families (Solanaceae, Leguminoseae and Brassicaeae, respectively) has enabled us to identify responses common to all three plant species and also to note some important differences.

It has been reported that *B. cinerea* produces hydrogen peroxide that is free to pass across lipid membranes in much the same way as water molecules. Hydrogen peroxide would be expected to oxidise Fe(II) to Fe(III) with the formation of the hydroxyl radical (HO•) through the Fenton reaction: $Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^- + HO\bullet$. Fe(II), however, is relatively rare in plant tissues, with Fe being transported predominantly as Fe(III) citrate and stored as ferritin, where the Fe occurs as Fe(III) oxyhydroxide polymers. Solubilisation of Fe from ferritin is accomplished readily by oxalate, a product of the metabolism of ascorbic acid by *B. cinerea*, and reaction of this Fe(III) with reductases or antioxidant molecules such as ascorbic acid yields the Fe(II) for the Fenton reaction. EPR spectroscopy was used to follow the changes in the oxidation states of transition metal ions such as Mn (II) and Fe(III) that can be readily detected in plant tissues and quantified through changes in signal intensity. Being transition metals, manganese and iron can exist in both EPR-detectable [e.g. Mn(II) and Fe(III)] and EPR-silent forms [e.g. Mn(III) and Fe (II)]. EPR therefore has the potential to provide information on the redox status of tissue samples and can also directly detect and characterise free radicals associated with complex molecules. We obtained evidence for effects of *B. cinerea* infection in pepper not previously recognised[2] by the detection of an unidentified, single-peak free radical

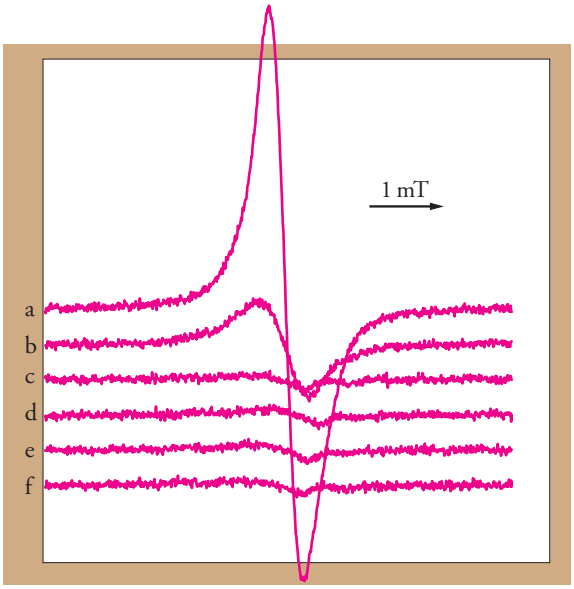

Figure 1 Typical EPR spectra (free radical region) of *B. cinerea*-inoculated pepper fruit. Samples were taken from increasing distances from the centre of the lesion: (a) centre of lesion, (b) edge of lesion, (c) 7mm from edge of lesion, (d) 15mm from edge of lesion, (e) 22mm from edge of lesion, (f) 30mm from edge of lesion.

(Fig. 1) and elevated levels of Fe(III) at g=4.3, especially within necrotic vascular traces that extend beyond the rotting lesion (Fig. 2). We obtained similar results in infected young leaves of French bean[3] and *A. thaliana*. There is therefore an indication that the plant deploys antioxidant systems in an attempt to redress the imbalance created by the pathogen. The levels of ascorbic acid decline appreciably in diseased tissues (Fig 3). When the spin trap POBN (α-(4-pyridyl-1-oxide)-N-*t*-butylnitrone) was introduced into samples from bean leaves, evidence was found also for an unstable free radical being involved in the disease process beyond the edge of the lesion (Fig. 4)

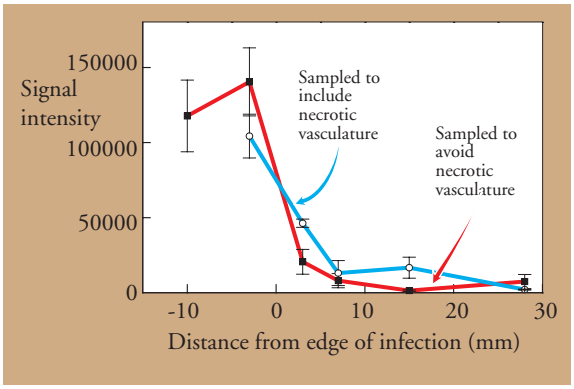

Figure 2 Intensity of EPR-detectable Fe(III) signal associated with specific lesions in *B. cinerea* infected pepper fruit.

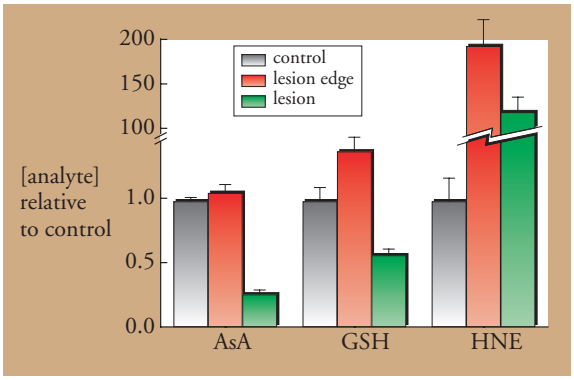

Figure 3 Ascorbic acid and glutathione depletion and accumulation of lipid peroxidation products from *B. cinerea* infection in *P. vulgaris*. AsA - ascorbic acid, GSH - reduced glutathione, HNE - 4-hydroxynonenal.

at the same time as a destabilisation of the ascorbate radical (Asc•) occurred. Similar results were found in pepper fruits[3].

Oxidative stress induced during the onset of plant disease results in degradation of cellular membranes by lipoxygenases and the peroxidation of lipids by peroxygenase. Linolenic acid from cell membranes is broken down to malondialdehyde (MDA) and 4-hydroxyhexenal (4-HHE) and linoleic acid to *n*-hexanal and 4-hydroxynonenal (HNE). These four aldehydes were quantified as their 2,4-dinitrophenylhydrazone derivatives by liquid chromatography-mass spectroscopy (LC-MS) to provide a measure of the lipid peroxidation that occurred in plant tissues as a result of infection.

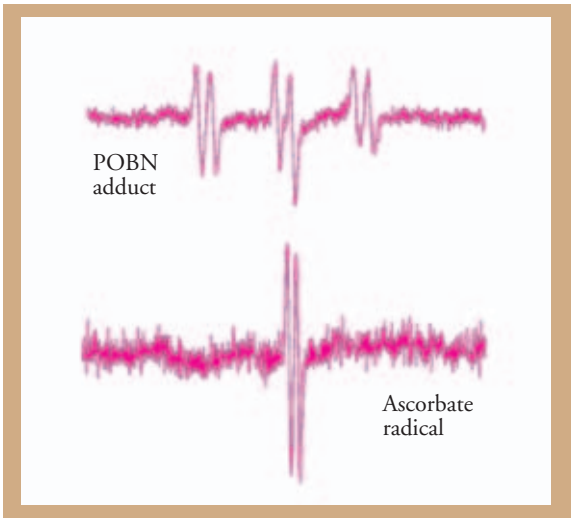

Figure 4 EPR detection of an unstable free radical (using the spin trap POBN) and the ascorbate radical in *B. cinerea* infected *P. vulgaris* leaves.

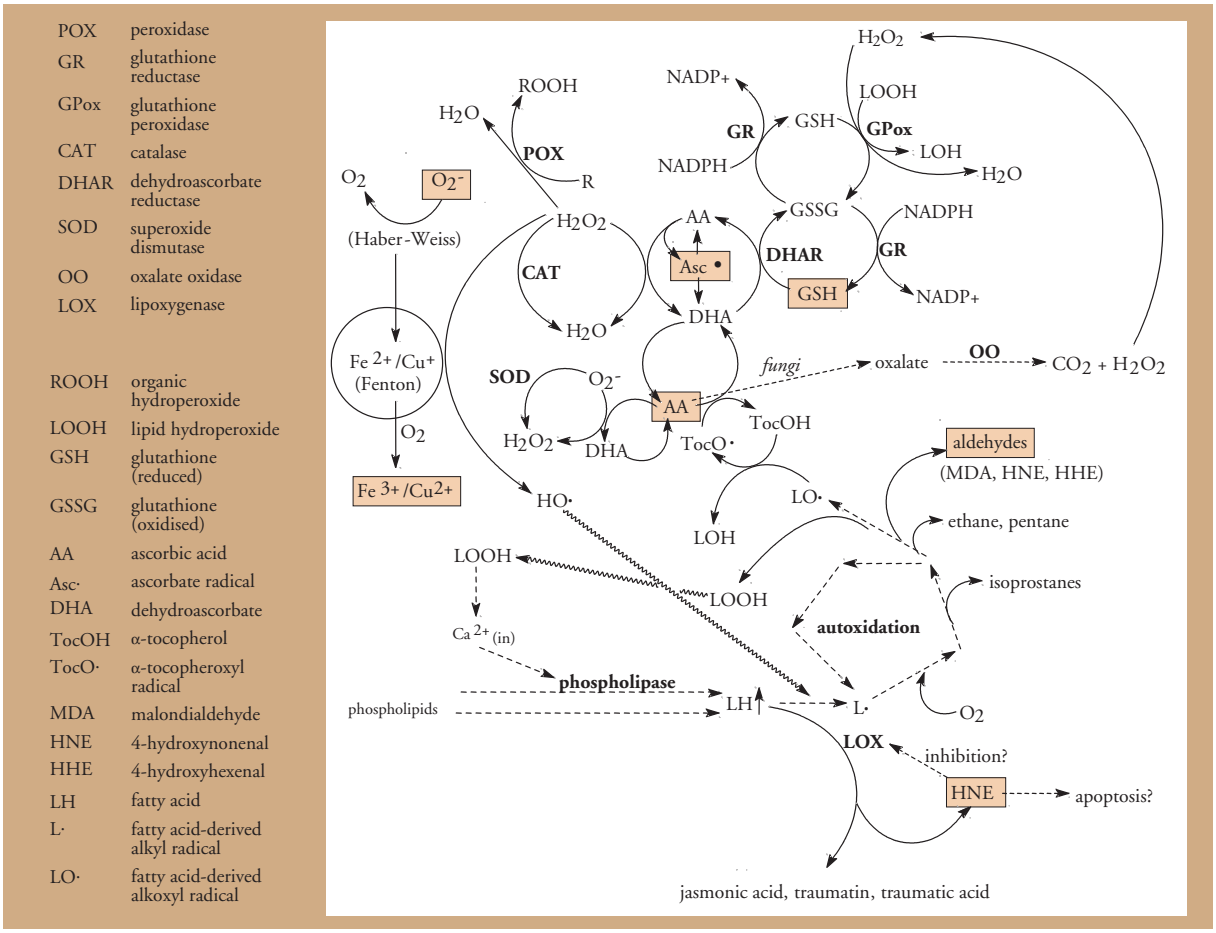

Figure 5 Relationship between oxidative processes associated with the infection process.

In sweet pepper fruits, elevated levels of HHE, HNE, MDA and *n*-hexanal were detected within the lesion and at the lesion margin. There was also some evidence of their extension into apparently healthy tissues[2]. The high levels of HHE and HNE found in diseased plant tissues is interesting because, in animal systems, they are known to be cytotoxic and genotoxic, reacting with amino and thiol groups in proteins and also with deoxyguanosine to form cyclic compounds at pH 7.4.

In leaves of four genotypes of *P. vulgaris* inoculated with *B. cinerea*, large increases in HNE and MDA were found adjacent to rotted tissue again indicating the powerful influence of this necrotroph on the host[4]. However, the expected accompanying increases in the levels of these aldehydic products of lipid peroxidation were not observed in *A. thaliana* inoculated under similar conditions, indicating that this species responds differently to infection compared with tissues from other plant families studied earlier. Thus, whilst *Arabidopsis* is an excellent model plant to study aspects of gene function and transcriptional regulation, there are limitations to the extent that information derived from *Arabidopsis* can automatically be related to plants in other families.

Massive depletion of ascorbic acid pools ahead of visible infection and generation of the EPR signals associated with the plant-pathogen interaction in representative species from three plant families, all indicate damage to the antioxidant mechanisms as an early event in the infection process (Fig. 5). It seems that once there is imbalance in oxidative processes, driven by the invading necrotrophic fungus, the latter is favoured and disease is the outcome.

References

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[2] Deighton, N., Muckenschnabel, I., Goodman, B.A. & Williamson, B. (1999). *The Plant Journal* **20**, 485-492.

[3] Muckenschnabel, I., Goodman, B.A., Deighton, N., Stewart, D., Lyon, G.D. & Williamson, B. (2001). *Protoplasma* **218**, 112-116.

[4] Muckenschnabel, I., Williamson, B., Goodman, B.A., Lyon, G.D., Stewart, D. & Deighton, N. (2001). *Planta* **212**, 376-381.