Botrytis cinerea perturbs redox processes as an attack strategy in plants

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Introduction

Oxidation reactions are a common feature of plants' responses to stress processes of both abiotic and biotic origin. These have been studied in fruits of *Capsicum annuum* and leaves of *Phaseolus vulgaris* (Fig.1) and *Arabidopsis thaliania* during formation of soft rots caused by *B. cinerea*.
B. cinerea produces H_2O_2 that passes freely across lipid membranes because, unlike water, it has no net dipole. H_2O_2 oxidises most forms of Fe(II) to Fe(III) with the formation of the hydroxyl radical (HO•):

$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^- + HO\bullet$ - the **Fenton** reaction

An exception is the Fe(II) nicotianamine complex (Fe-NA), which is resistant to oxidation, and is stable in normal plant tissues. However, at pH < ~7.5, Fe-NA is destabilised relative to Fe(II) citrate, and this is an active Fenton-reaction agent. Furthermore, the major form of Fe in plants, ferritin, (where it occurs as Fe(III) oxyhydroxide polymers) is readily solubilised by oxalate, a product of metabolism of ascorbic acid by *B. cinerea*.

Reaction of Fe(III) with reductases or antioxidant molecules, such as ascorbic acid (AA), yields Fe(II) and (often) free radicals:

$Fe(III) + AA \rightarrow Fe(II) + AA\bullet$

where AA• is the ascorbate radical. Fe(II) is also generated by superoxide ($O_2^{\bullet -}$):

$Fe(III) + O_2^{\bullet -} \rightarrow Fe(II) + O_2$ - the **Haber-Weiss** reaction

Thus infection of plant tissue by *B. cinerea* leads to redox cycling of Fe, the generation of free radicals (FR) and FR reaction products, and the depletion of antioxidant molecules.

Mononuclear Fe(III) complexes were measured directly in plant tissues by EPR spectroscopy, which was also used to detect and characterise associated free radicals. In addition, the aldehydes malondialdehyde (MDA) and 4-hydroxyhexenal (4-HHE) (from linolenic acid breakdown) and *n*-hexanal and 4-hydroxynonenal (4-HNE) from linoleic acid were quantified by liquid chromatography-mass spectroscopy (LC-MS) along with the antioxidants, AA and glutathione.

Figure 1
Spreading soft rot Botrytis Lesion four days after inoculation.

Material and Methods

Plant tissues and pathogen inoculation.

Leaves of intact plants of *P. vulgaris* and *A. thaliana* were inoculated with 14-day-old conidial suspensions of *B. cinerea* isolate B05.10 under conditions that gave rise to spreading soft rots [3,4]; other work used detached fruits of *C. annuum* inoculated with another isolate [1,2]. Samples for analysis were taken from within lesions, at the lesion margins and at varying distances into uninfected tissue, and compared with samples from non-inoculated controls held under the same conditions.

EPR spectroscopy.

Spectra were obtained with a Bruker ESP300E X-band spectrometer using an ER4103TM cylindrical cavity. Samples were held in a quartz 'finger dewar' at –196°C and spectra recorded with parameters used by Muckenschnabel *et al.*[3] The generation of unstable free radicals was monitored by a spin-trapping technique[2] involving infiltration of the spin trap 4-POBN into the plant tissue. 4-POBN reacts with unstable free radicals (in competition with indigenous free radical scavengers) to produce radical adducts that can be characterised.

LC-MS.

Analyses were carried out on a Finnigan MAT SSQ710C single quadrupole mass spectrometer with APCI interface. Quantification of 4-HHE, MDA, 4-HNE, *n*-hexanal, AA and reduced glutathione (GSH) was performed as described previously[3].

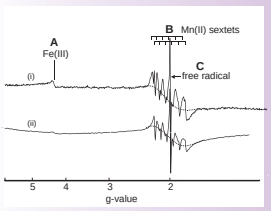

Figure 2
Typical EPR spectrum from plant tissue. (i) rotted tissue from leaf of *A. thaliana* inoculated with *B. cinerea* and (ii) control *A. thaliana* leaf. Signal A, mononuclear Fe(III) complexes; B, Mn(II) complexes, and C, free radical. Ferritin is indicated by the dashed line.

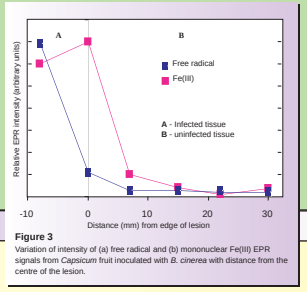

Figure 3
Variation of intensity of (a) free radical and (b) mononuclear Fe(III) EPR signals from *Capsicum* fruit inoculated with *B. cinerea* with distance from the centre of the lesion.

Results

In all samples, the EPR spectra (Fig. 2) showed a large increase in intensity of signals from a free radical and a mononuclear Fe(III) complex (Fig. 3) associated with infection. In Phaseolus leaves infiltrated with 4-POBN, free radical adducts were detected in rotted tissue and in apparently healthy tissue at positions remote from the lesion margins, whereas uninfected leaves showed the ascorbate free radical (Fig. 4). A similar result was seen in *C. annuum fruit*, where the 4-POBN adduct signals were more intense in the region of vascular tissues[2] (Fig. 5). The observation of the radical adducts in infected plants indicates a decreased ability of tissue to compete with the spin trap for free radicals; i.e. the free radical scavenging ability of the host has been compromised by the pathogen.

Massive depletion of AA pools, and some depletion of GSH were also associated with the plant-pathogen interaction. In *A. thaliana* (but not *P. vulgaris*) leaves, this depletion of ascorbic acid extended to the uninfected regions of inoculated leaves (Fig. 6).

Elevated levels of 4-HHE, 4-HNE, MDA and n-hexanal were detected within the lesion and at the lesion margin in *C. annuum* fruits inoculated with *B. cinerea*; there was also some evidence of their extension into apparently healthy tissues. Leaves of *P. vulgaris* also showed large increases in HNE and MDA in green areas adjacent to rotted tissue, but there was no increase in the levels of these aldehydic products of lipid peroxidation in similar samples from *A. thaliana* (Fig. 6).

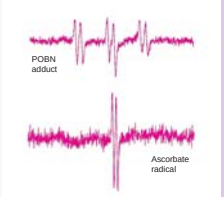

Figure 4
EPR spectra from *P. vulgaris* leaves infiltrated with the spin trap 4-POBN. (a) rotted tissue resulting from infection with *B. cinerea*, (b) healthy tissue.

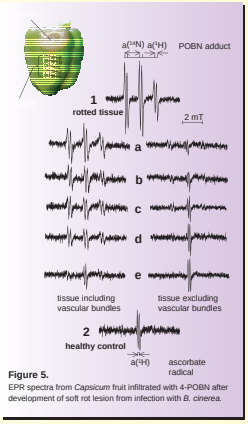

Figure 5.
EPR spectra from *Capsicum* fruit infiltrated with 4-POBN after development of soft rot lesion from infection with *B. cinerea*.

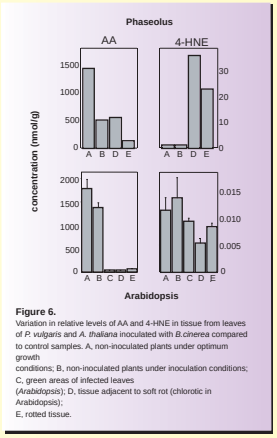

Figure 6.
Variation in relative levels of AA and 4-HNE in tissue from leaves of *P. vulgaris* and *A. thaliana* inoculated with *B.cinerea* compared to control samples. A, non-inoculated plants under optimum growth conditions; B, non-inoculated plants under inoculation conditions; C, green areas of infected leaves (*Arabidopsis*); D, tissue adjacent to soft rot (chlorotic in Arabidopsis); E, rotted tissue.

Discussion

Massive oxidative damage, seen as changes in the status of intercellular iron, the generation of lipid peroxidation products and damage to the antioxidant machinery, occurs in host tissues in advance of colonisation by *B. cinerea*. The formation of 4-HHE and 4-HNE is interesting because they are cytotoxic and genotoxic in animal tissues; at pH 7.4 they react with amino and thiol groups in proteins, and with deoxyguanosine to form cyclic compounds.

Our data also suggest that the plants' antioxidant systems, such as the ascorbate-glutathione cycle (Fig 7), were inadequate to cope with the oxidative processes driven by *B. cinerea*, under the environmental conditions (*i.e.* high humidity) used in these experiments. This necrotrophic pathogen, like the related *Sclerotinia sclerotiorum*[5], probably utilises the plant's ascorbic acid pool to generate H_2O_2 via oxalate (Fig. 7). Both pathogen and host deploy similar oxidative defence systems and at the interface there must be a fine balance between them dependent on the relative sensitivity of each partner to the toxic compounds generated (Fig. 8).

The amounts of products in the plant-pathogen interaction vary from one system to another. This is illustrated by the responses of *Phaseolus* and *Arabidopsis* leaves to infection by *B. cinerea*. Compared to control samples, the green areas of infected leaves of *P. vulgaris* showed massive increases in the levels of lipid peroxidation products and small decreases in the levels of the measured antioxidants. In contrast, similar samples from *A. thaliana* showed massive depletion of antioxidants, but no increase (small decrease) in the measured lipid peroxidation products.

Although this work has examined changes in key products of oxidative processes during lesion spread, further biochemical studies are still needed to follow the dynamics of oxidative enzyme pathways associated with host defences.

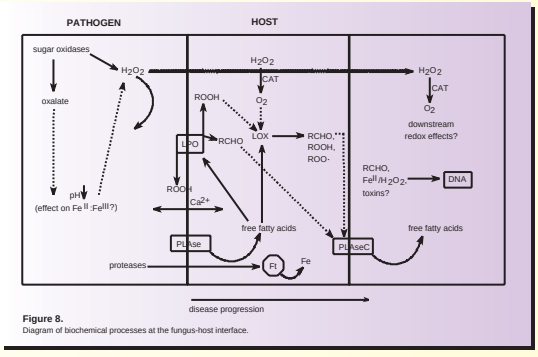

Figure 8.
Diagram of biochemical processes at the fungus-host interface.

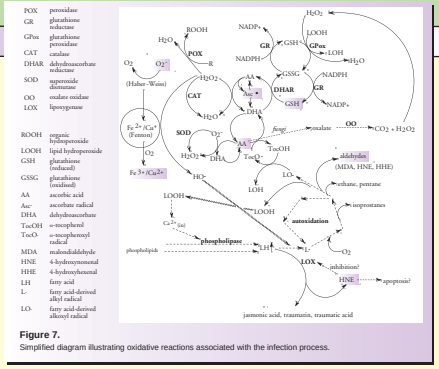

Figure 7.
Simplified diagram illustrating oxidative reactions associated with the infection process.

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