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Oxidative stress responses during cassava post-harvest physiological deterioration

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Abstract

A major constraint to the development of cassava (*Manihot esculenta* Crantz) as a crop to both farmers and processors is its starchy storage roots' rapid post-harvest deterioration, which can render it unpalatable and unmarketable within 24–72 h. An oxidative burst occurs within 15 min of the root being injured, that is followed by the altered regulation of genes, notably for catalase and peroxidase, related to the modulation of reactive oxygen species, and the accumulation of secondary metabolites, some of which show antioxidant properties. The interactions between these enzymes and compounds, in particular peroxidase and the coumarin, scopoletin, are largely confined to the vascular tissues where the visible symptoms of deterioration are observed. These, together with other data, are used to develop a tentative model of some of the principal events involved in the deterioration process.

Abbreviations: ACMV, African cassava mosaic virus; AFLP, amplified fragment length polymorphism; CAT, catalase; cDNA, complementary deoxyribonucleic acid; CIAT, International Centre for Tropical Agriculture; Cu/ZnSOD, copper/zinc superoxide dismutase; DAB, 3,3-diaminobenzidine tetrahydrochloride; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FeSOD, iron superoxide dismutase; FW, fresh weight; GUS, β-glucuronidase; HPTLC, high-performance thin-layer chromatography; HR, hypersensitive response; IEF-PAGE, isoelectric focusing polyacrylamide gel electrophoresis; MAS, marker-assisted selection; MeJa, methyl jasmonate; MnSOD, manganese superoxide dismutase; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); NBT, nitroblue tetrazolium; PAL, phenylalanine ammonia-lyase; PCD, programmed cell death; PCR, polymerase chain reaction; POX, peroxidase; PPD, post-harvest physiological deterioration; QTL, quantitative trait loci; ROS, reactive oxygen species; RT, room temperature; SAR, systemic acquired resistance; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase

Introduction

Upon harvesting, cassava (*Manihot esculenta* Crantz) starchy storage roots suffer a rapid deterioration that renders them unpalatable and unmarketable within 24–72 h, depending on the cultivar and environmental conditions. This deterioration is a purely physiological process, though microbial deterioration can set in subsequently, and is known as post-harvest physiological deterioration (PPD). Within traditional village societies, where cassava roots are harvested, consumed or processed for local need as required, PPD does not present a serious problem. However, with increasing urbanisation in less developed countries, the entry of

rural farmers into the cash economy and processing on a larger or industrial scale (Balagopalan, 2002; Westby, 2002), times and distances between field and consumer or processor have increased, and PPD has become a major constraint to the development of cassava for farmers, processors and consumers alike as a result of the discounting, waste and added costs that it causes (Janssen and Wheatley, 1985; Wenham, 1995; Ndunguru *et al.*, 1998; Beeching, 2001; Vlaar *et al.*, 2001). An aspect of these problems is that urban consumers turn to alternative, often imported, carbohydrate foods, which negatively impacts on rural and national economies. Therefore, research directed towards introducing resistance to PPD, or delaying the response, is considered a priority by international bodies such as the Food and Agriculture Organisation of the United Nations (FAO) and the Cassava Biotechnology Network (Wenham, 1995). Increasing the shelf-life of cassava to one or two weeks, either by conventional breeding or via biotechnology, is considered an achievable long-term objective by these bodies.

Processing into more stable traditional or industrial products avoids the problem of PPD. While this is practical at the village or local scale, at a larger scale time and distance between harvesting and processing can cause PPD to become a serious problem by varying the input quality to the industrial processes. The exclusion of oxygen by storing and transporting the roots in plastic sacks, while delaying PPD, has not been adopted, largely due to the cost involved. However, coating individual roots with paraffin wax has been successfully adopted for the export of cassava for food to markets or countries where the roots can command a high price. But this is not practical for the bulk treatment of such a low-cost commodity in producing countries, neither is storage in controlled atmospheres with reduced oxygen content, or the freezing of roots. Pruning cassava stems 10–20 cm above the ground two weeks before harvest does reduce susceptibility to PPD (Kato *et al.*, 1991) but, unfortunately, negatively affects eating and starch qualities, and so has not been adopted (C.C. Wheatley, personal communication).

Breeding has been used successfully in cassava, for example to improve yield or African cassava mosaic virus (ACMV) resistance, but it has yet to be applied to improving cassava with respect to its PPD response (Jennings and Iglesias, 2002). However, the high heterozygosity of cassava combined with its limited flowering makes breeding a complex and challenging task especially for some traits. There exists a correlation between PPD and high dry matter content (a desirable characteristic), which could be difficult to separate through a conventional breeding programme (Iglesias *et al.*, 1996). In addition, PPD is a complex multigenic trait with a strong environmental interaction (Cortés *et al.*, 2002). These data imply that conventional breeding for reduced PPD, though possible, may not be straightforward.

In a heterozygous clonally propagated crop such as cassava, genetic improvement via biotechnological approaches offers an alternative strategy to conventional breeding, especially for such a complex trait as PPD. A biochemical and molecular understanding of the process of PPD is being developed (Beeching *et al.*, 1998; Buschmann *et al.*, 2000a, b; Han *et al.*, 2001; Reilly *et al.*, 2001), several transformation systems are now available (Puonti-Kaerlas, 1998) and the technology has been applied to modify useful phenotypes in the cassava root (Taylor *et al.*, 2001b; Zhang *et al.*, 2003) including features related to PPD (Taylor *et al.*, 2001a). In addition to the direct manipulation of the plant's genetic makeup, aspects of this technology can produce invaluable information and tools, such as the production of molecular genetic maps of the cassava genome, quantitative trait loci (QTLs) mapping and marker-assisted selection (MAS), which can assist and accelerate breeding programmes (Frova *et al.*, 1998; Knapp, 1998). These latter approaches are already being applied to cassava (Fregene *et al.*, 1997; Cortés *et al.*, 2002) Therefore, biotechnology has the power not only to dissect PPD in cassava and thus permit its detailed understanding, but also to provide the tools to modify the trait directly via genetic modification or indirectly via MAS. Analysis of the environmental components of the response, and/or screening of existing genetic diversity with respect to PPD within primary, secondary and tertiary gene pools, also have the potential to provide useful information on PPD that might be relevant for its modulation; however, such approaches have not been adequately explored.

The initial visual symptoms of PPD are blue-black discolorations of the vascular tissues that spread from wound sites caused by harvesting or handling (Booth, 1976), preceded and accompanied by a strong fluorescence of the cassava tissue under ultra violet light (Wheatley, 1982). Microscopy reveals that coloured occlusions and tyloses block xylem vessels (Rickard *et al.*, 1979). In parallel to these visual events are increases in respiration (Hirose, 1986), changes in lipid composition (Lalaguna and Agudo, 1989), secondary metabolite accumulation (Rickard, 1981; Uritani

et al., 1983; Wheatley and Schwabe, 1985; Sakai and Nakagawa, 1988), the synthesis of the phytohormone ethylene (Hirose *et al.*, 1984), and increases in the activity of a range of enzymes including phenylalanine ammonia-lyase (PAL), acid invertase, catalase, dehydrogenases, peroxidases and polyphenol oxidase (Rickard, 1981; Tanaka *et al.*, 1983). Evidence from cycloheximide inhibition of protein synthesis (Uritani *et al.*, 1984) and other data (Beeching *et al.*, 1998) confirm that PPD is an active, rather than a degenerative, process involving changes in gene expression and protein synthesis.

The secondary metabolites that accumulate during PPD include diterpenic and phenolic compounds, some of which have anti-microbial and antioxidant activity (Sakai and Nakagawa, 1988; Buschmann *et al.*, 2000a, b). Peaks of reactive oxygen species (ROS) and increased activity of enzymes that modulate ROS are detected during deterioration (Reilly *et al.*, 2000, 2001). These data, coupled with the observation that the exclusion of oxygen prevents PPD, are strong circumstantial evidence that ROS and the enzymes and compounds that modulate them play important roles during PPD. Further evidence for this conclusion comes from screening the International Centre for Tropical Agriculture's (CIAT) cassava germplasm collection, which showed a relationship between the content of carotenoids in the root, above a certain threshold concentration (5 mg per kg fresh weight), and reduced PPD response (Chavez *et al.*, 2000).

Directed and random sequencing of cDNA clones corresponding to genes that are expressed during PPD, together with cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis, shows that these include proteins and enzymes that are involved in signal transduction pathways, phytohormone synthesis, are themselves anti-microbial or are involved in the synthesis of anti-microbial, antioxidant or otherwise defensive compounds, are involved in senescence or aspects of programmed cell death, are concerned with modulating ROS, and others that are involved in the synthesis of cell wall components, and proteins that are themselves components of cell walls (Beeching, 2001; Han *et al.*, 2001; Huang *et al.*, 2001; Reilly *et al.*, 2001). Components of these cDNAs and their corresponding genes could serve as potential tools in genetic constructs for exploring the nature of the PPD response and even to approach modulating that response in transgenic cassava. Certainly, a cassava PAL promoter in transgenic cassava, while it shows developmental expression in vascular tissue elsewhere in the plant, is also up-regulated during PPD in those vascular tissues of the harvested root where the deterioration symptoms are observed (Taylor *et al.*, 2001a). Therefore, while this promoter is not necessarily a strong candidate for the modification of the PPD response, it serves to illustrate the kind of gene component that could be. Several of these characterised clones have been added to the molecular map of the cassava genome produced at CIAT, thereby providing some named genes related to PPD amongst the largely anonymous molecular markers used to date (Cortés *et al.*, 2002). The Family K mapping population (used for the molecular map) has been screened twice in different agro-ecological zones for its PPD response in order to identify QTLs that contribute to PPD. These data show that PPD is a heritable response to which several regions of the genome contribute. However, it is subject to environmental factors that severely complicate its analysis. The parents of Family K were chosen primarily for their contrasting resistance responses to ACMV and *Xanthomonas axonopodis* (Fregene *et al.*, 1997), while they do show some differences in their PPD response, these were not sufficient to permit clear-cut progeny analysis, especially when confused by high environmentally induced variability.

These data suggest that PPD shows strong parallels in cassava roots to wound responses in other more fully studied plants, and highlight the roles of ROS and senescence phenomena in these post-harvest events. Plant wounding produces or induces the production of signalling molecules that initiate the wound response; this response includes the production of defensive compounds and enzymes, the preparation of the plant for the potential extension of wounding, and wound repair that is followed by the inhibition of signals (Bowles, 1998). While these aspects of the wound response are present in the harvested cassava root, the wound repair and the resultant down-modulation of the signals are inadequate, which leads to continuous cascades of wound responses that spread throughout the root; it is this that is observed as PPD. It is interesting that wounded roots that remain attached to the plant are capable of normal wound repair (Mwenje *et al.*, 1998), suggesting that either efficient wound repair of the detached roots has been lost during the millennia since domestication, or that, because the root serves no function once detached from the plant, the trait has been lost during evolution as of no biological purpose.

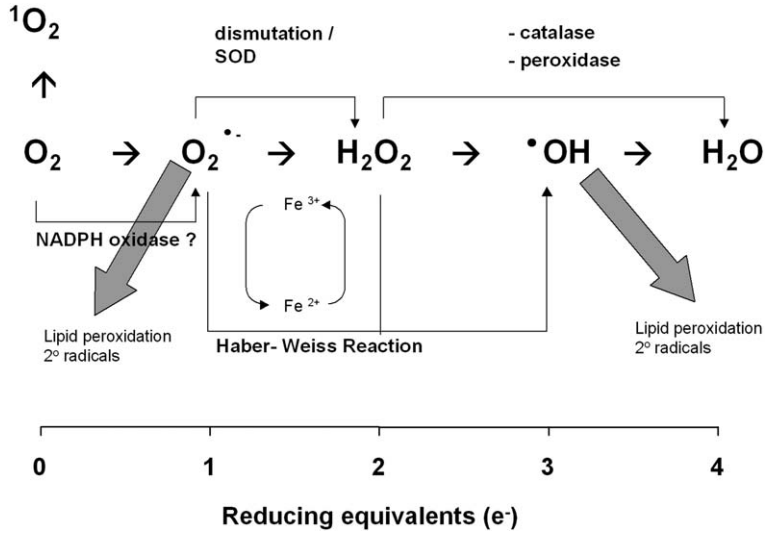
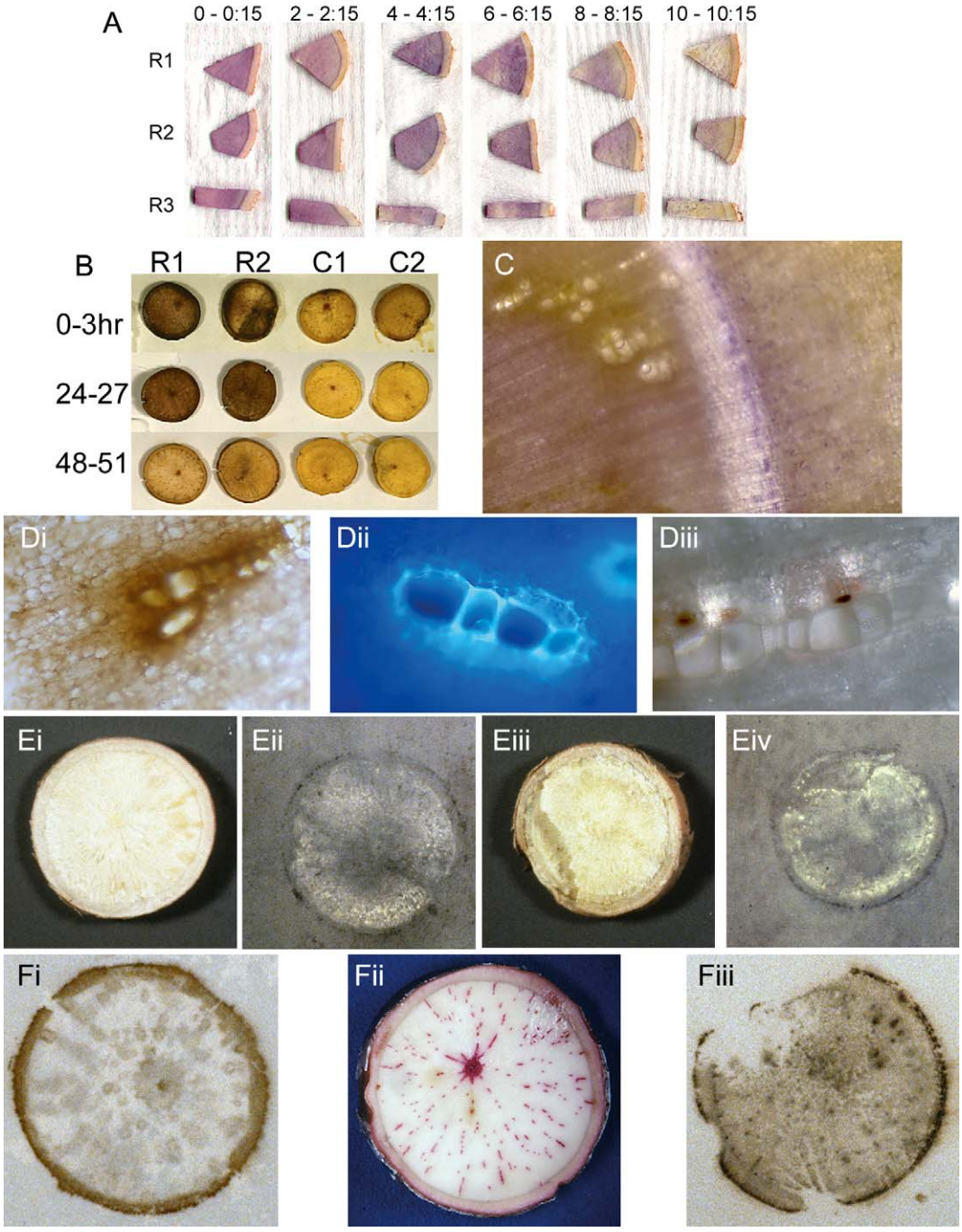


Figure 1. Reactive oxygen species (ROS) formed from molecular oxygen, and likely inter-conversion pathways that may occur in plants. Singlet oxygen ($^1O_2$) is a highly reactive form of di-oxygen (O_2) in which one of the un-paired electrons of ground-state di-oxygen is promoted to an orbital of higher energy. The superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH) are formed by one-electron reductions of molecular di-oxygen. Cellular defences such as superoxide dismutase (SOD), catalase and peroxidase serve to scavenge O_2^- and hydrogen peroxide (H_2O_2), thereby preventing their participation in the formation of hydroxyl radical (OH) via the iron catalysed Haber-Weiss reaction. A plasma membrane NADPH oxidase has been proposed to catalyse the initial formation of superoxide from molecular oxygen, although alternate mechanisms have also been suggested (Bolwell *et al.*, 2002).

ROS refer to reactive molecules that result from the reduction of molecular di-oxygen. Whilst molecular di-oxygen is relatively non-reactive and non-toxic, it becomes reactive once its electron structure is altered. Although many ROS are oxygen radicals, i.e. contain an unpaired electron, several, such as singlet oxygen and hydrogen peroxide, are not. The main forms of ROS known to exist in plants and the major inter-conversion pathways between them are summarised in Figure 1. Secondary radicals such as the alkoxyl radical ($RO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and organic hydroperoxides (ROOH) may be formed in planta by lipid peroxidation initiated either enzymatically (e.g. lipoxygenase) or by ROS such as superoxide or the hydroxyl radical (Larson, 1995). Although neither $O_2^{\iota-}$ nor H_2O_2 is highly reactive at physiological concentrations, toxicity in vivo arises from their role as substrates in the iron catalysed Haber-Weiss reaction. The hydroxy radical (OH^{ι}) formed via this reaction and its derivatives are amongst the most reactive species known, and react indiscriminately with cellular macromolecules causing lipid peroxidation, protein denaturation and deoxyribonucleic acid (DNA) damage.

In plant systems, the major initial sources of ROS during normal metabolism are the production of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) via electron 'leakage' from electron transport chains (e.g. photosystems I and II, the mitochondrial electron transport chain, electron transport chains located in the ER, membrane, peroxisomes and nuclear envelope), and the production of singlet oxygen $^1O_2$ during photosynthesis by transfer of excitation energy from triplet chlorophyll to molecular di-oxygen (Bartosz, 1997). Under stress conditions, increased ROS formation often occurs through perturbation of such metabolic paths, and cellular damage arising from environmental stresses is often caused by oxygen radicals. A large body of research has focused on the controlled production of ROS in plant defence, particularly of superoxide and hydrogen peroxide, and especially during the hypersensitive response (HR) and systemic acquired resistance (SAR). This 'oxidative burst' is defined as a rapid production of superoxide and/or hydrogen peroxide in response to external stimuli (Wojtaszek, 1997). The ROS generated during the oxidative burst play several complex and overlapping roles in facilitating plant defence. Essentially, these may be summarised as (1) cell wall strengthening, (2) induction of defence related genes, and (3) triggering of host cell death. An oxidative burst during defence responses to bacterial, viral and nematode pathogens or elicitors, host cell wall derived elicitors such as oligogalacturonide, wounding, mechanical stress and


A
0 - 0:15
2 - 2:15
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8 - 8:15
10 - 10:15
R1
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C2
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C
Di
Dii
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Ei
Eii
Eiii
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Fi
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Figure 2. A. *In situ* detection of O_2^- production in wounded cassava storage roots (cv. MCol 22). Three root samples were examined for superoxide production over a time course of 10 h. Areas of superoxide accumulation are indicated by the formation of a blue reaction product with NBT. Time after injury is indicated at the top of the panel in hours and hours : minutes. R1, root 1; R2, root 2; R3. root 3. B. *In situ* detection of H_2O_2 production in wounded cassava storage roots (cv. MCol 22). Areas of H_2O_2 production are indicated by the formation of a brown reaction product with 3,3-diaminobenzidine tetrahydrochloride (DAB). Control reactions for each time point were infiltrated with DAB (2 mg/ml) and 10 mM ascorbate and did not show brown coloration. Time after injury is indicated at the left of the panel. R1, root 1; R2, root 2; C1, control treatment root 1; C2, control treatment root 2. C. Localisation of superoxide in hand-cut cassava storage root sections (cv. MCol 22) examined by light microscopy. High levels of superoxide in individual cambium cells stain blue-purple. D. Localisation of (i) H_2O_2, (ii) scopoletin and (iii) peroxidase activity in cassava storage root (cv. Mcol22). Areas of H_2O_2 accumulation are indicated by the formation of a brown reaction product with DAB (panel Di), Scopoletin accumulation was detected by fluorescence microscopy (panel Dii), and peroxidase activity was detected by the formation of a brown reaction product with guiacol (panel Diii). E. Tissue print detection and localisation of catalase activity in the cassava storage root of MCol 22. Areas of catalase activity are indicated by the formation of a clear area on a dark background. Ei, cassava root slice immediately after harvest (T = 0 days); Eii, tissue print on nitrocellulose paper showing catalase activity (T = 0 days); Eiii, cassava root slice 4 days after harvest; Eiv, tissue print on nitrocellulose paper showing catalase activity, 4 days after harvest. F. Tissue print detection and localisation of peroxidase activity in cassava roots (cv. MCol 22). Fi, tissue print on nitrocellulose paper showing peroxidase activity in a transverse slice of a non-deteriorated root (T = 0 days); Fii, tissue slice from Fi stained with phloroglucinol: lignified and suberised tissues stain red; Fiii, tissue print showing peroxidase activity in a deteriorated root 5 days after harvest.

heat or cold shock has been described, and it has been suggested that the oxidative burst may form part of a general stress defence pathway in plants (Scott *et al.*, 1999).

A number of enzymatic and non-enzymatic defences have evolved to detoxify ROS and/or to prevent the formation of highly damaging and reactive forms such as the hydroxyl radical. Superoxide dismutase (SOD, superoxide:superoxide oxidoreductase, EC 1.15.1.1) catalyses the dismutation of superoxide to hydrogen peroxide and oxygen, and is one of the primary ROS-scavenging enzymes found in aerobic organisms. In plants, three types of SOD are distinguished on the basis of the metal cofactor at the active site: manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD) and iron superoxide dismutase (FeSOD). Catalase (H_2O_2: H_2O_2oxidoreductase, EC 1.11.1.6) is a tetrameric heme-containing enzyme, which serves to break down hydrogen peroxide. Catalase has been proposed to play a role in mediation of signal transduction involving hydrogen peroxide as a second messenger, possibly via a mechanism involving salicylic acid (Leon *et al.*, 1995). Peroxidases catalyse one electron oxidations using hydrogen peroxide as the electron acceptor and a wide variety of organic or inorganic substrates as the electron donor. The classical secretory or guiacol peroxidases (donor:H_2O_2 oxidoreductase, EC. 1.11.1.7) are monomeric, haem-containing enzymes. They are often further subdivided in the literature into cationic (pI 8.1–11), moderately anionic (pI 4.5–6.5) and anionic (pI 3.5–4) peroxidases (Abrahams *et al.*, 1996).

Several lines of evidence suggest that the PPD response in cassava roots is an enzymatically mediated oxidative process, since molecular oxygen is required and treatments to reduce oxygen access and/or inhibit enzyme activity can inhibit the response (Averre, 1967; Noon and Booth, 1977; Rickard, 1982; Beeching *et al.*, 1998). In addition, decreases in the non-enzymatic antioxidants β-carotene and ascorbate have been reported during PPD, and a high root carotene content is associated with reduced susceptibility to PPD (Iglesias *et al.*, 1995).

In this paper we examine the role of ROS and the enzymes that mediate their interconversion during the post-harvest physiological deterioration of the cassava root, and approach a tentative model for PPD.

Materials and methods

Plant material

Cassava plants were grown in the tropical glasshouse at the University of Bath at 22–28 °C, relative humidity 40–80% and a minimum light period of 12 h per day under daylight, supplemented with 400 W Philips high-pressure sodium lights when necessary. Storage roots were harvested after nine months' growth. Alternatively, roots were obtained from cassava grown in the field at CIAT, Cali, Colombia.

Induction of PPD

Immediately after harvest proximal and distal ends of the storage roots were removed and two transverse V-shaped cuts made along the length of the root. Exposed proximal and distal ends of the root were covered with Parafilm and roots were stored at ambient temperature.

In our hands this system gives an even progression of PPD symptoms throughout the root.

In situ detection and localisation of ROS

Freshly harvested roots were injured by cutting into transverse slices. For each time point, in situ production of $O_2^{\bullet -}$ was detected by the formation of a blue precipitate with nitroblue tetrazolium (NBT, Sigma) (Vallélian-Bindschelder *et al.*, 1998). H_2O_2 was detected by the formation of a brown precipitate with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Thordal-Christensen *et al.*, 1997). At each time point samples were infiltrated with DAB (2 mg/ml) under vacuum and incubated at room temperature (RT) for 3 h. Controls were co-infiltrated with 10 mM ascorbate (a scavenger of H_2O_2) and did not show any brown coloration. For microscopic observation, thin hand-cut sections were prepared and $O_2^{\bullet -}$ detected (Barcelo, 1998). Hydrogen peroxide was detected as above.

Quantification of hydrogen peroxide

A modification of the method of Warm and Laties (1982) was used. In order to remove coloured compounds, root extracts (0.05 g fresh weight (FW)) were homogenized in 1.5 ml of 5% metaphosphoric acid and passed twice through a Dowex basic anion-exchange resin (Sigma). Emitted photons were measured in a luminometer (Micro Lumat Plus, E and G Berthold) using volumes of 195 μl of 0.2 M Tris-HCl buffer pH 8.5, 25 μl of 0.1 mM luminol, and 5 μl of root extract. For each sample, luminescence was initiated by automated injection of 25 μl of 5 M potassium ferricyanide, and emitted photons were measured over a time period of 15 s after a delay of 5 s. A standard curve was obtained using known volumes of 10 mM H_2O_2 in 5% metaphosphoric acid.

Extraction and analysis of root secondary metabolites

Root samples were ground in liquid nitrogen, suspended in ethanol and blended with an Ultra Turrex blender. Samples were stored at RT for 30 min and then filtered through Whatman No. 1 filter paper. Samples and washings were transferred to a vacuum flask and evaporated. Each sample was re-suspended in ethanol and dissolved by sonication for 30–60 s before filtration through a nylon filter (HPLC technology, pore size 0.22 μm). Root extracts were analysed by high-performance thin-layer chromatography (HPTLC) on silica gel 60 HF_{254} plates (Merck) with a solvent system of chloroform/ethyl acetate/methanol (2: 2: 1, v/v). Secondary metabolite bands were detected by fluorescence at 254 nm and 366 nm. Potential antioxidant bands were detected with 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent (Takao *et al.*, 1994).

Nucleic acid methods

Cassava cDNA clones were isolated from a PPD-related cDNA library constructed in λgt10 from mRNA isolated from storage roots of cassava cultivar MNga 1 at 48 h after harvest (Beeching *et al.*, 1997), by screening at low stringency (1× SSC at 50 °C). The isolation of the catalase clone, MecCAT1, has been described previously (Reilly *et al.*, 2001). Probes for the isolation of the superoxide dismutase (MecCuZnSOD) and peroxidase (MecPX2) cDNA clones were amplified by polymerase chain reaction (PCR) from the cDNA library with the oligonucleotide primers 5′-GATGAICTTGGIAAGGGICATG-3′ (MecCuZnSOD) and 5′-ACGAAGCAGTCGTGG AA-3′ (MePX1), designed on the basis of conserved motifs, and either of the λgt10 universal forward and reverse primers (New England Biolabs). DNA sequencing was done on an Applied Biosystems ABI 337 automated sequencer.

Total RNA for northern hybridisations was extracted from storage roots or leaves (Reilly *et al.*, 2001). Total RNA for pre-harvest pruning treatments was extracted from storage roots of plants which had been pruned by removal of the stem at a height of ca. 30 cm from the ground two weeks prior to harvest. Control samples were prepared from similar plants that had not been subjected to the pruning treatment. For the ethylene treatment freshly harvested root slices were incubated in the ethylene-generating compound ethephon (Sigma) (0.02% in sterile water) for 24 h in the dark. Control slices were incubated in water alone. For methyl jasmonate (MeJa) treatment root slices were incubated for 24 h in the dark in MeJa (Sigma) (500 μM in 0.1% ethanol). Control slices were incubated in 0.1% ethanol. Total RNA (10 μg) was electrophoresed on a 1% agarose gel containing formaldehyde and blotted onto nylon membrane (Hybond N plus, Amersham). Hybridisation was carried out with ^{32}P-labelled cDNA clones as probes at 65 °C overnight in 0.5 M phosphate buffer pH 7.2, 1% skimmed milk, 7% SDS. The membrane was washed in 1× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature for 1–2 min, twice in 0.2× SSC, 0.2% SDS for 15 min at 65 °C and then visualized

with a FLA 5000 phosphoimager (Fuji) with a BAS-MS imaging plate. All other molecular methods were standard (Sambrook *et al.*, 1989).

Protein extraction and analysis

At each time point total protein was extracted according to standard procedures and protein content determined (Stoscheck, 1990). Isoelectric focusing polyacrylamide gel electrophoresis (IEF PAGE) was carried out on broad-pI-range ampholine PAG plates pH 3.5–9.5 (Amersham Pharmacia) with the Multiphor II electrofocusing LKB BROMMA 2117 system (Pharmacia Biotechnology). SOD isoforms were detected on an overlay gel (Manchenko, 1994). Peroxidase isoforms were detected with guiacol (H_2O_2 0.1% v/v, aqueous guiacol 10 mM, in 50 mM phosphate buffer pH 5.2) or aminoantipyrine (50 mM phosphate buffer pH 6, 100 mM DHBS (3,5-dichloro-2-hydroxybenzenesulfonic acid), 10 mM 4-aminoantipyrine, 100 mM H_2O_2). Peroxidase isoforms with activity towards scopoletin were detected according to Gutierrez-Mellado *et al.* (1996).

Tissue print and in situ *detection of enzyme activity*

Tissue prints were prepared according to Varner and Ye (1994). Catalase activity was detected as previously described (Reilly *et al.*, 2001). Peroxidase activity was detected according to Peyrado *et al.* (1996). For *in situ* detection and localisation of peroxidase activity, thin hand-cut sections were placed on a glass slide and immersed in 0.1% H_2O_2 in 10 mM aqueous guiacol. After 5 min the sections were examined by light microscopy.

Results

Reactive oxygen species are produced during deterioration

We report here the occurrence of a wound-induced 'oxidative burst' measured as both superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), in the cassava storage root. Superoxide production was detected within 15 min after injury, declining to low levels 8–10 h later (Figure 2A). Hydrogen peroxide was detected within 3 h after injury of the roots (Figure 2B). Peak levels were observed at 24 h after injury before a gradual decline (Figure 3). Peak levels attained (ca. 5 μmol per gram FW) were comparable with reported levels for a mechanically induced oxidative burst in other plant systems (Cazale *et al.*, 1998).

To characterise the response further, we examined ROS production histochemically. Diffuse purple/blue staining indicative of superoxide formation was observed in the root parenchyma (Figure 2C). The cambium area of tissue sections showed a ring of intense staining associated with individual cells within the cambium. These strongly staining cells were primarily located at the exterior part of the cambium just underlying the cortex. In the cassava storage root this part of the cambium gives rise to the secondary phloem (Cabral *et al.*, 2000). Given a recent proposed model for wound induced ROS production via a systemin-mediated pathway (Orozco-Cardenas and Ryan, 1999), this location is interesting since systemin is phloem-mobile. H_2O_2 was initially localised mainly to the cortical parenchyma and internal storage parenchyma just underlying the cambium, where it was especially associated with xylem vessels and surrounding packaging parenchyma cells (Figure 2Di).

Accumulation of ROS-scavenging secondary metabolites during root deterioration

A number of plant phenolic compounds that could potentially act as antioxidants have been shown to accumulate in the cassava storage root after harvest. These include the fluorescent coumarin compounds scopoletin, scopolin and esculetin as well as two unidentified scopoletin and esculetin conjugates, and the flavonoids (+)catechin and gallocatechin (Tanaka *et al.*, 1983; Uritani *et al.*, 1984; Buschmann *et al.*, 2000a, b). A TLC-based method with the coloured radical compound DPPH reagent was used to detect the accumulation of secondary compounds capable of scavenging oxygen radicals in injured cassava storage roots, and to assess the free-radical scavenging potential of reference compounds known to be produced during PPD (Figure 4). These data indicated that the flavan-3-ol compounds (+)gallocatechin, and (+)catechin, and the coumarin compounds scopoletin and esculetin were capable of acting as free-radical scavengers, whilst the coumarin scopolin showed no free-radical-scavenging ability. Separation of ethanolic root extracts indicated the presence of at least five secondary metabolites of retention factors R_f 0.68, R_f 0.56, R_f 0.34, R_f 0.23 and R_f 0.04 that could function *in vivo* as free-radical-scavenging antioxidants. Based on the R_f values for this solvent system three of these were identified as the coumarin scopoletin (R_f

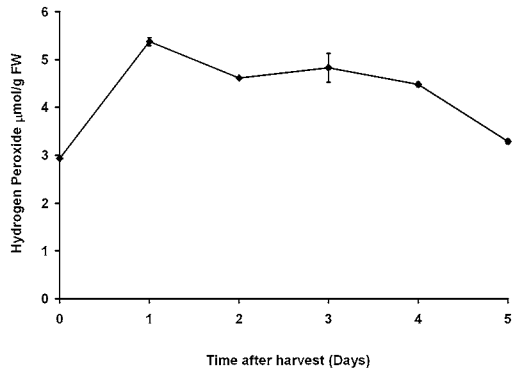

Figure 3. Quantification of hydrogen peroxide production in wounded cassava storage roots (cv. MVen 77) over a 5-day time course. Each point represents the mean of three roots examined (mean ± SD).

0.56), and the flavonoids gallocatechin (R_f 0.23) (Figure 4) and rutin (R_f 0.04) (Buschmann *et al.*, 2000c). These data indicate that the cassava storage root contains a range of easily oxidised compounds that could participate as electron donors in enzymatic or non-enzymatic oxidation reactions during the post-harvest period.

Expression and activity of enzymes that modulate ROS

The major initial sources of ROS in plant systems are $O_2^{\bullet-}$ and H_2O_2, which may be produced in a 'controlled' manner via the oxidative burst or as a result of 'leakage' from electron transport chains. Dismutation of $O_2^{\bullet-}$ to $H_2O_2$and O_2 may occur spontaneously or may be catalysed by the enzyme SOD (Figure 1). Our data indicates the presence of four SOD isoforms in the cassava storage root (Figure 5). No obvious changes in isoform pattern or intensity were observed over a 5-day post-harvest time course, suggesting that regulation of SOD isoforms does not play a significant role in the development of PPD. In addition, a cassava root Cu/Zn SOD cDNA clone, MecCuZnSOD (GenBank accession number AF426273) isolated from a root PPD-related cDNA library, was expressed at similar levels in storage root, leaf and petiole (Figure 6).

Increases in enzyme activity of both peroxidase and catalase, the two key enzymes involved in turnover of H_2O_2, occur during PPD (Beeching *et al.*, 1998). We have isolated and characterised cassava storage root catalase and peroxidase cDNA clones, MecCAT1 (GenBank accession number AF170272) and MecPX2 (AY033386), which were up-regulated during PPD (Figure 7). The MecCAT1 transcript was

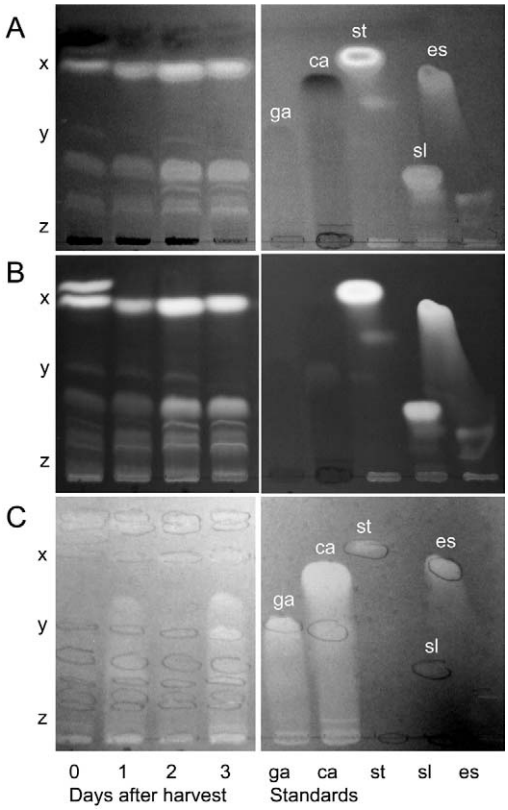

Figure 4. TLC separation and detection of potential antioxidant compounds in the cassava storage root (cv. MCol 22). The TLC plates were viewed under 254 and 366 nm UV light (panel A and B respectively) and after treatment with DPPH (panel C). Clear areas after treatment with DPPH reagent in panel C indicate free-radical-scavenging activity. Standard compounds were ga, gallocatechin; ca, (+) catechin; st, scopoletin; sl, scopolin; es, esculetin, which are indicated just above the corresponding bands in panels A and C. Five bands showing free radical scavenging activity in the cassava root are indicated on the left: band x (Rf 0.56) was identified as scopoletin; band y (Rf 0.23) was identified as gallocatechin; band z (Rf 0.04) was identified as rutin; bands a (Rf 0.68) and b (Rf 0.34) were not identified.

predominantly expressed in the storage root, although expression was also detected in leaf tissue (Figure 6). Interestingly, MecPX2 showed storage root-specific expression with no expression detected in petiole or leaf. Given the apparent root specificity of this peroxidase clone and its rapid transcript up-regulation in response to injury of the roots, isolation of the cognate promoter may be of interest for further studies.

Peroxidase and catalase activities were localised in the cassava root during PPD by light microscopy and/or tissue printing techniques. Catalase activity was distributed throughout the root parenchyma (Figure 2Ei–iv). Increased activity in less susceptible

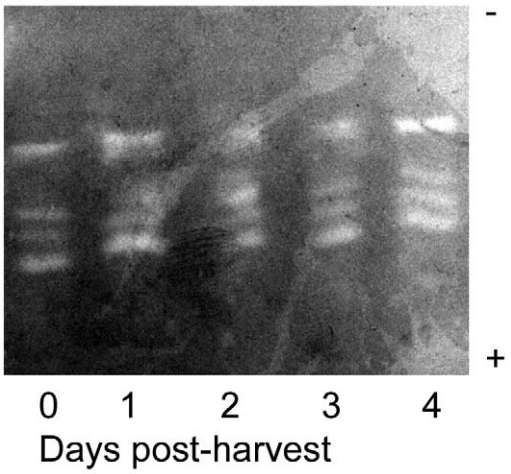

Figure 5. Expression of superoxide dismutases in the cassava storage root after harvest. Total protein was extracted from storage roots of cultivar MPer 337 at 0, 1, 2, 3 and 4 days after injury. Isoforms were separated by IEF PAGE and detected according to Materials and methods.

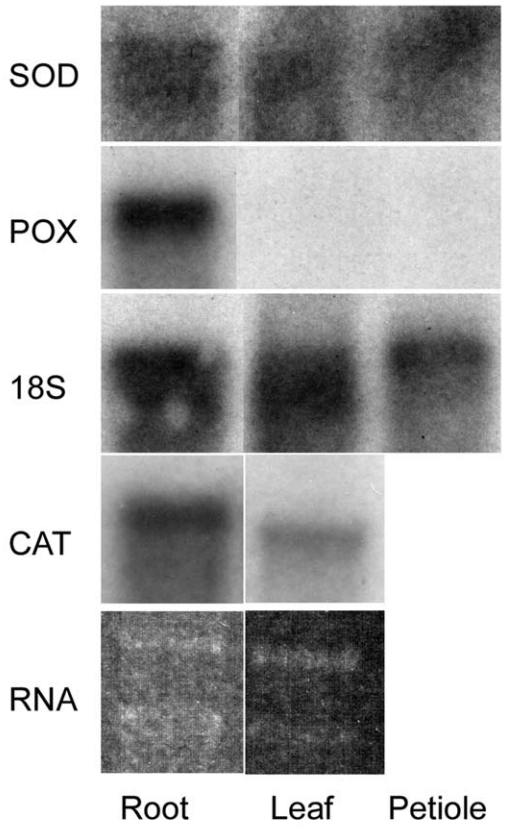

Figure 6. Northern blot analysis of transcript accumulation in different tissues of the cassava plant (cv. MDom 5). Total RNA (10 μg) extracted from root, leaf or petiole immediately after harvest, was electrophoresed, transferred onto nylon membranes and hybridised with ^{32}P-labelled probes. SOD, MecCuZnSOD transcript expression; POX, MecPX1 transcript expression; CAT, MecCAT1 transcript expression; 18S, 18sRNA; RNA, ribosomal RNA.

cultivars has been reported (Reilly *et al.*, 2001). In freshly harvested roots, peroxidase activity was localised to the epidermis, cortex and xylem tissues of the storage root, with little activity detected in the root parenchyma (Figure 2Fi). This peroxidase activity corresponded well with the areas of suberin and lignin, which stain red with phloroglucinol (Figure 2Fii). In deteriorated roots, showing visible symptoms of PPD, peroxidase activity was more extensive than in freshly harvested roots having spread throughout the root parenchyma (Figure 2Fiii). Cytochemical observations using light microscopy confirmed the change in peroxidase localisation observed by tissue printing. Peroxidase activity was localised to particular cells of the xylem parenchyma directly adjacent to the xylem vessels (Figure 2Diii). This localisation is of particular interest given cytological studies indicating the initial site of production of the coloured occlusions associated with PPD in the xylem parenchyma cells, followed by movement into the xylem vessels via pit areas (Beeching *et al.*, 1998).

Peroxidase isoforms are likely to be of particular interest regarding PPD since total peroxidase activity increases during the response (Marriott *et al.*, 1978) whilst less susceptible cultivars exhibit lower levels of peroxidase activity during the post-harvest period (Campos and Decarvalho, 1990). In addition, the peroxidase substrate, scopoletin, shows a transient accumulation in cassava roots (Buschmann *et al.*, 2000b) and has been proposed as a key component of the deterioration response (Beeching *et al.*, 2002). IEF PAGE showed a range of peroxidase isoforms with differing expression patterns during the post-harvest period in the cassava storage root (Figure 8). Two cationic isoforms showing apparent up-regulation during PPD were detected in both cultivars examined. The MecPX2 cDNA clone, which has a predicted pI of 8.9 is likely one of the two cationic peroxidase isoforms observed. The anionic peroxidases were more varied between the two cultivars, with MCol 22 and MPer 337 showing different predominant anionic peroxidase isoforms. A number of peroxidase isoforms with activity towards scopoletin have been detected in other plants (Gutierrez-Mellado *et al.*, 1996), and further IEF PAGE experiments indicated that at least two cassava root anionic peroxidase isoforms showed activity towards scopoletin (Figure 9).

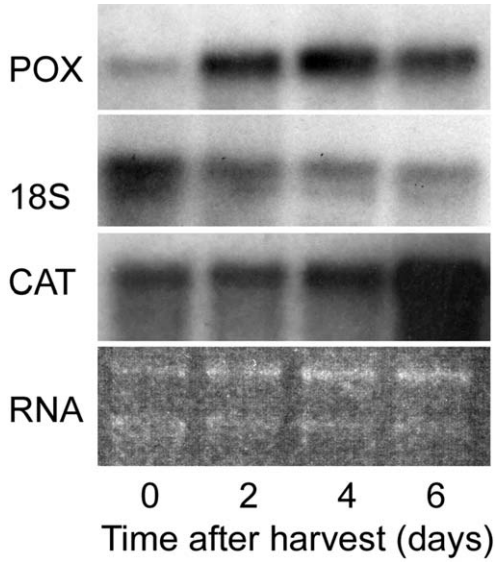

Figure 7. Northern blot analysis of catalase and peroxidase transcript accumulation during post-harvest storage of cassava roots (cv. MCol 22). Total RNA (10 μg) extracted from storage roots at various times after harvest was electrophoresed, transferred onto nylon membranes and hybridised with ^{32}P-labelled probes. POX, MecPX1 transcript expression; CAT, MecCAT1 transcript expression; 18S RNA; RNA, ribosomal RNA.

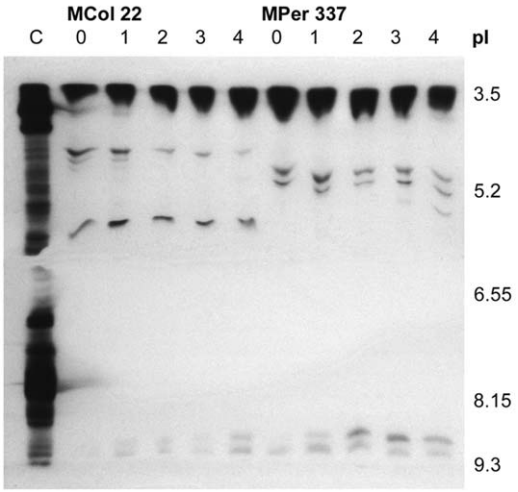

Figure 8. Expression of peroxidase isoforms in cassava storage root after harvest. Total protein was extracted from storage roots of cultivars MCol 22 and MPer 337 at 0, 1, 2, 3 and 4 days after injury. Isoforms were separated by IEF PAGE and detected with guiacol (H_2O_2 0.1% v/v, aqueous guiacol 10 mM, in 50 mM phosphate buffer pH 5.2). C is horseradish peroxidase.

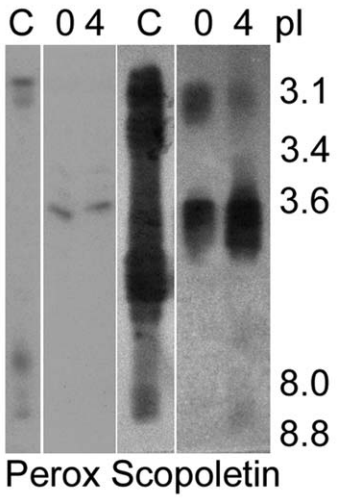

Figure 9. Detection of peroxidase isoforms with activity towards scopoletin in cassava storage root after harvest. Total protein was extracted from storage roots of cv. MCol 22 and at 0, and 4 days after injury. Duplicate gels, including horseradish peroxidase (C), were used to separate isoforms by IEF PAGE, and then stained for peroxidase activity, with aminoantipyrine, and activity towards scopoletin according to Materials and methods.

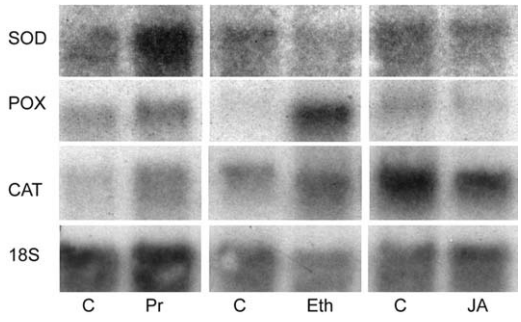

Figure 10. Northern blot analysis of catalase (CAT) MecCAT1, peroxidase (POX) MecPX1 and superoxide dismutase (SOD) MecCu/ZnSOD transcript accumulation in response to various treatments. Total RNA (10 μg) extracted from treatment and control roots (cv. MCol 22) was electrophoresed, transferred onto nylon membranes and hybridised with ^{32}P-labelled probes. SOD, MecCuZnSOD transcript expression; POX, MecPX1 transcript expression; CAT, MecCAT1 transcript expression; 18S RNA. C, control treatments; Pr, pre-harvest pruning treatment; Eth, ethephon treatment (0.02% ethephon in sterile water); JA, methyl jasmonate treatment (500 μM in 0.1% ethanol).

To date little is known about possible signals that may lead to the onset or modulation of PPD. The plant hormone ethylene is produced within ca. 6 h after injury of cassava storage roots (Beeching *et al.*, 1998) and could potentially play a signalling role. In other plant systems MeJa has been implicated in wound signal transduction. Northern hybridisation experiments indicated that catalase MecCAT1 and peroxi-

dase MecPX2 transcripts were up-regulated by treatment with the ethylene-generating compound ethephon, whilst MecCu/ZnSOD showed little change relative to control treatments (Figure 10). None of the three ROS-scavenging enzyme transcripts was up-regulated in response to MeJa treatment. Cassava storage roots from plants that had been pruned prior to harvest show decreased susceptibility to PPD, although the reason for this reduced susceptibility are not clear (Kato *et al.*, 1991). Our data indicate that catalase MecCAT1 transcript levels were higher in roots from pruned plants than non-pruned controls (Figure 10). MecCu/ZnSOD transcript levels were also somewhat higher in roots from pruned plants relative to non-pruned controls, whilst MecPX2 transcript levels showed little change.

Discussion

We have attempted to draw together data presented in this paper, together with other work from our laboratory and elsewhere, in order to develop a model for some of the processes that occur in the cassava storage root during PPD. It has been proposed that the blue/black vascular streaking observed as PPD may result from peroxidase-mediated oxidation of scopoletin (Wheatley and Schwabe, 1985), since oxidation of scopoletin by peroxidases is known to give rise to a bluish product of unknown structure. Localisation data (Figure 2Di–iii) indicate that within the root parenchyma all required components of the reaction, namely scopoletin, H_2O_2, and peroxidase activities, are initially localised to the vicinity of the xylem vessels where vascular streaking symptoms occur. Moreover, peroxidase isoforms with activity towards scopoletin are present in the cassava storage root (Figure 9). Interestingly, PAL promoter-β-glucuronidase (GUS) fusion studies show that PAL is likewise expressed in the region of the xylem tissues of the storage root and is up-regulated during PPD in transgenic cassava plants (Taylor *et al.*, 2001a). Scopoletin is produced via the phenylpropanoid pathway and PAL represents the key entry point into phenylpropanoid metabolism. Thus up-regulation of PAL in the vicinity of the xylem vessels at the onset of PPD may function to synthesise the substrate for the vascular streaking reactions.

At the molecular level the development of PPD is evidently a complex phenomenon involving multiple components. Based on current knowledge, a speculative model for the processes that occur during PPD and of potential interactions that may occur between the different components is shown in Figure 11. We have reported here the occurrence of a wound-induced oxidative burst in injured cassava storage roots, the accumulation of secondary metabolites, some of which show antioxidant properties, and the altered regulation of genes related to the modulation of reactive oxygen stress. The up-regulation of genes related to defence and wound healing such as PAL, β-glucanase and hydroxyproline-rich glycoproteins (HRGPs), and of proteases, protease inhibitors and other genes that have been implicated in senescence or programmed cell death responses in other plant systems, have been reported (Beeching, 2001; Han *et al.*, 2001; Huang *et al.*, 2001; Reilly, 2001; Taylor *et al.*, 2001a).

Of the ROS-scavenging enzymes, catalase MecCAT1 and peroxidase isoforms, including the root peroxidase transcript MecPX1, were up-regulated during the post-harvest storage period. Differences in catalase levels at both the enzyme and the transcriptional level have been reported (Reilly *et al.*, 2001), with levels of MecCAT1 transcript and overall catalase activity being more pronounced in less susceptible cultivars. In addition, roots from pruned plants, which show reduced susceptibility to PPD, showed higher levels of MecCAT1 transcript accumulation than roots from non-pruned controls (Figure 10), raising the possibility that higher catalase levels may exert a protective effect. A similar role for catalase has been demonstrated during senescence of muskmelon fruits (Lacan and Baccou, 1998). In a comparative study on two muskmelon varieties showing contrasting storage life, higher sustained levels of catalase activity were found in the long storage life variety. Enzyme assay and tissue printing studies have indicated that overall peroxidase activity is lower in roots of less susceptible cassava cultivars (Campos and Decarvalho, 1990; Reilly, 2001). Taken together, these data suggest that roots from less susceptible cultivars or from pruned plants may efficiently utilise catalase to scavenge H_2O_2 produced after wounding, resulting in the formation of molecular oxygen and water:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

In non-pruned plants or more susceptible cultivars, where catalase levels are relatively lower and peroxidase levels relatively higher, a significant proportion of H_2O_2 scavenging could occur via peroxidase-mediated reactions requiring the participation of cellular components, including scopoletin, as an electron

donor:

$$H_2O_2 + 2AH \rightarrow 2H_2O + 2A$$

Thus, relatively higher levels of peroxidase would lead to increased oxidation of scopoletin, observed as increased vascular streaking.

Of the defence-related genes, PAL shows up-regulation within 24 h after injury in cassava storage roots (H. Li, Y. Han, K. Reilly and J.R. Beeching, submitted). As the key entry point into phenylpropanoid metabolism, altered PAL expression could affect diverse pathways including the synthesis of wound healing components such as lignin and suberin, the synthesis of wound- or stress-related secondary metabolites including scopoletin, and signalling compounds such as salicylic acid.

Aspects of wound healing responses do occur in the cassava storage root and storage roots can produce a wound periderm under conditions of high temperature and humidity (curing), albeit slower than in other root crops. However, under normal storage conditions wound healing is not adequate to repair the harvested root and thus prevent the PPD response. Cassava root HRGPs, an important class of protein involved in wound healing, are expressed during PPD but do not show up-regulation until three days after harvest by which time PPD is well set in (Han *et al.*, 2001). It is likely that the failure of wound healing and sealing has the effect of driving gene expression away from re-establishment of homeostasis and towards continued defence responses.

Sequencing of PPD-related ESTs and transcript derived fragments (cDNA-ALFPs) from cassava storage roots undergoing PPD indicates that around 6% of genes expressed during PPD can be classified as PCD (programmed cell death)-associated genes (Beeching, 2001; Huang *et al.*, 2001). These data raise the intriguing possibility that PPD of cassava storage roots may include components of PCD as is seen in other plant defence responses such as the hypersensitive response (HR). Indeed, several parallels could be drawn between PPD and the HR. Both processes are active ones and can be inhibited by cycloheximide, increases in ethylene production have been noted before the onset of HR cell death, as have the accumulation of fluorescent coumarin components including scopoletin, up-regulation of PAL and β-glucanases, carotenoid degradation, membrane breakdown and increases in peroxidase and catalase activity (Pontier *et al.*, 1999). Cell death certainly does occur during PPD of the cassava storage root; however, no studies have been carried out to determine if such cell death reflects necrotic cell death or a form of PCD. It has been proposed by several groups that PCD is a default pathway in both plants and animals, and it may be that severance of the root from the plant and associated wounding results in the triggering of such a pathway.

With respect to signals leading to the onset of PPD to date only two potential signal components that could lead to the modulation of PPD responses have been identified. Firstly, the plant hormone ethylene was detected within 6 h of injury of cassava storage roots, and lower ethylene levels were detected in less susceptible roots, although pre-harvest pruning did not prevent ethylene synthesis (Hirose, 1986), leading to the speculation that ethylene may play a role in signal transduction during PPD. Secondly, we report here the occurrence of a wound-induced oxidative burst measured as both superoxide anion and H_2O_2 accumulation. The role of H_2O_2 as a signalling molecule in plants, mediating responses to abiotic and biotic stresses is becoming increasingly clear. Recently microarray data have indicated that around 1% of the *Arabidopsis* transcriptome is regulated by H_2O_2 (Desikan *et al.*, 2001). H_2O_2 produced via the oxidative burst could potentially act to modulate PPD-related gene expression in the cassava storage root, as well as participating in a range of oxidative processes ranging from peroxidase-mediated oxidation of cellular components to cell wall cross-linking of HRGPs as part of the healing or curing response. Northern blot hybridisation data presented here indicate that catalase MecCAT1 and peroxidase MecPX1 are up-regulated by ethylene. MecPAL1 (GenBank accession number AY036011) may be regulated by both ethylene and H_2O_2, since analysis of the PAL promoter used in promoter-GUS fusion studies in transgenic cassava (Taylor *et al.*, 2001a) indicates the presence of putative H_2O_2- and ethylene-responsive motifs.

Treatments known to inhibit PPD could act to impede the PPD progression pathway as proposed in Figure 11 in various ways. One formed the superoxide anion (O_2^-) could lead directly to cell membrane damage via the iron catalysed Haber-Weiss reaction. Spontaneous or SOD-mediated dismutation of O_2^- to H_2O_2 could lead to induction of PPD-related gene expression and to oxidation of cellular components including peroxidase-mediated oxidation of scopoletin.

Exclusion of molecular oxygen (for example modified atmospheres, wax dipping) would act to prevent entry of molecular oxygen, thereby preventing its conversion into O_2^-. Under conditions of high tem-

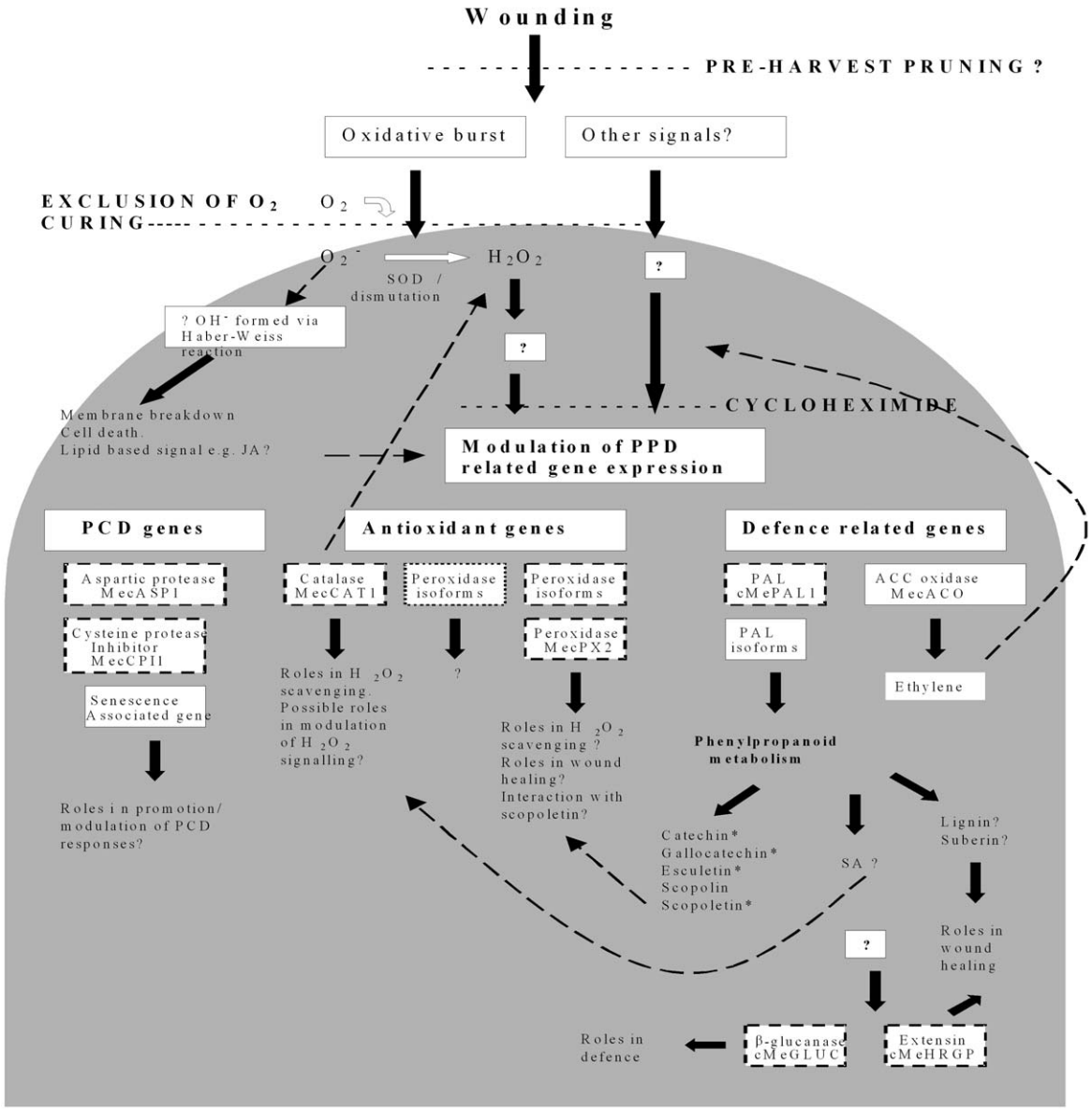

Figure 11. Schematic model representing processes that may occur during post-harvest physiological deterioration of the cassava storage root. Genes or proteins known to be expressed during PPD are indicated. Those known to be up-regulated are indicated in dashed boxes, those known to be down regulated are indicated in dotted boxes. Possible interactions between components in the model are indicated by dashed line arrows. Unknown components are indicated with a question mark. Possible points where treatments known to inhibit PPD may act are indicated in capital letters. Of the secondary phenolic compounds known to accumulate in the root after injury, those that are capable of acting as antioxidants are marked with an asterisk. Genes that are expressed in the later stages after injury (after 48 h) are indicated towards the base of the figure.

perature and humidity the injured cassava root can form a wound periderm (curing) that inhibits development of PPD. Under such conditions of high humidity one would expect a higher proportion of intracellular spaces to be filled with water, thus effectively excluding oxygen.

High-temperature treatments (such as dipping roots in hot water at 60 °C for 45 min) and low-temperature storage (Averre, 1967; Booth, 1976) also inhibit PPD and would be expected to impair enzyme activity perhaps preventing vascular streaking via inhibition of peroxidase activity and/or by inhibiting enzymes responsible for the conversion of molecular oxygen to O_2^-. Similarly, cycloheximide treatment would act to prevent synthesis of enzymes required for the development of PPD.

The nature of the 'protectant' signal resulting from pre-harvest pruning is as yet unknown. Increased lignification, changes in starch and sugar composition, and reduced activities of PAL, acid invertase, and peroxidases, have previously been reported in roots from pruned plants (Data *et al.*, 1984; Kato *et al.*, 1991). Similarly, our data indicates that pre-harvest pruning can result in changes in gene expression that may serve to protect the storage roots from subsequent injury.

It is clear that PPD is a complex phenomenon involving both genetic and environmental factors. With respect to the considerable environmental induced variation in PPD it is tempting to speculate that such variation may reflect something as simple as the micronutrient status of the plant. For example, levels of available iron could affect progression of the Haber-Weiss reaction; similarly both the secretory peroxidases are haem-containing enzymes whose activity may be affected by relative levels of iron available within the plant.

The proposed model for PPD put forward here is not expected to be complete or wholly accurate; however, it is our hope that it may serve to stimulate interest in an important problem in an important food crop.

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