Extracellular production of reactive oxygen species in response to abiotic stress in seeds

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Abstract

Reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the hydroxyl radical ($^{\bullet}OH$) can damage essential biomolecules including nucleic acids, proteins and lipids, causing damage to various cellular components. However, ROS also participate in signalling networks that are essential in plant stress responses, and also in the regulation of growth and development. Given the apparent importance of ROS, it is surprising that very little of their beneficial aspects have been researched in seeds and seedlings.

Using desiccation sensitive ("recalcitrant") seeds of sweet chestnut (*Castanea sativa* Mill.) and desiccation tolerant ("orthodox") seeds of garden pea (*Pisum sativum* L.), the production of ROS was investigated during germination and seedling development, and in response to abiotic stress. Putative extracellular ROS-producing enzymes in both species were characterised to elucidate mechanisms of ROS production.

Desiccating *C. sativa* seeds led to viability loss while the intracellular antioxidant glutathione became increasingly oxidised. Wounding and desiccation induced extracellular ROS production in *C. sativa* embryonic axes and *P. sativum* seedling axes. A pivotal role for extracellular peroxidases in producing $O_2^{\bullet -}$ as a stress response became evident in both species as well as for the development of *P. sativum* seedlings. Wounding also induced amine oxidases in *P. sativum* embryonic axes to produce a burst of H_2O_2 that was essential for $O_2^{\bullet -}$ production. Lipoxygenases were identified as putative $O_2^{\bullet -}$-producing enzymes that may contribute to stress signalling in response to wounding. Treating desiccation-stressed material with H_2O_2 improved seed germination, seedling vigour and the establishment of secondary root growth. In conclusion, cell wall peroxidases, amine oxidases and lipoxygenases may work in synergy to produce $O_2^{\bullet -}$required for stress signalling. Such extracellular ROS produced by seeds appear to be important signalling components involved in wound and desiccation response, regeneration and growth.

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Abbreviations

$^{\bullet}OH$	Hydroxyl radicla
$^{1}O_2$	Singlet oxygen
2-Br	2-bromoethylamine
$^{3}O_2$	Ground state molecular oxygen
AA	Ascorbic acid
ABTS	2,2'-azino-bis 3-ethylbenzo-thiazoline-6-sulphonic acid
AOX	Amine oxidases
B1	Proteins loosely associated to the cell wall
B2	Proteins bound by hydrophobic interactions
B3	Proteins bound by ionic bonds
B4	Un-removable cell wall proteins
C	Cytosolic fraction
DAB	3,3'-diaminobenzidine
DPI	Diphenylene iodonium
DTT	Dithiothreitol
DW	Dry weight
ECPOX	Extracellular peroxidase
$E_{RSSR/2RSH}$	Half-cell reduction potential of LMW thiol – disulphide redox pairs
ESR	Electron spin resonance
FW	Fresh weight
G6PDH	Glucose-6-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectroscopy
GSH	Reduced glutathione
GSSG	Glutathione disulphide
H_2O_2	Hydrogen peroxide
$HO_2^{\bullet}$	Perhydroxyl radical
HPLC	High performance liquid chromatography
ISTA	International Seed Testing Association
LMW	Low-molecular-weight

LOX	Lipoxygenases
LSD	Least Significant Difference
MAPK	Mitogen activated protein kinases
MW	Molecular weight
NAD(P)H	Reduced β-nicotinamide adenine dinucleotide (phosphate)
NaN_3	Sodium azide
NBT	Nitroblue tetrazolium
$O_2^{\bullet -}$	Superoxide anion radical
P-buffer	50 mM sodium phosphate buffer at pH 7.0
PAGE	Polyacrylamide gel electrophoresis
PCD	Programme cell death
POX	Peroxidases
PUFA	Polyunsaturated fatty acids
RH	Relative humidity
ROS	Reactiove oxygen specie(s)
SE	Standard error
SOD	Superoxide dismutase
TG	Total germination
TMB	3,3',5,5'-tetramethyl-benzidine
WC	Water content

Chapter 1

Introduction

The unavoidable exposure of plants to environmental stress can lead to the enhanced production of potentially damaging reactive oxygen species (ROS). Historically, research into ROS-related plant stress responses focused on antioxidants and mechanisms that prevent "oxidative stress", a term to describe the loss in cellular redox balance potentially leading to damage (Sies, 1991). However, roles for ROS in signalling networks and physiological events increasingly emerged that have now added a new angle to redox biology research (Foyer and Noctor, 2005). As Halliwell (2006) stated "free radicals are not all bad, nor antioxidants all good". Put another way, antioxidants prevent ROS from causing damage, while allowing them to carry out their functions. This thesis characterises the production of ROS by seeds and seedlings in response to various stresses. The introduction begins, as the initial focus of the ROS research did, on the destructive and detrimental nature of ROS. The subsequent sections describe the nature and function of antioxidants and how they can ameliorate oxidative stress. Finally, the relevance of ROS to seed biology is explained together with the aims of this thesis.

1.1 Reactive oxygen species

In non-photosynthetic tissue, such as seeds, electrons leaked from mitochondrial electron transport chains are responsible for the majority of intracellularly produced ROS. The terminal step of respiration, catalysed by cytochrome oxidase (EC 1.9.3.1), uses $^3O_2$ as the final electron acceptor. The $^3O_2$ molecule accepts four electrons from NADH, thereby producing 2 molecules of H_2O, oxygen in its most reduced state. However, electrons are only transferred one at a time because of the parallel spins of the oxygen molecules outermost non-bonding electrons (Halliwell and Gutteridge, 1999). Between 98 – 99 % of $^3O_2$ consumed is fully reduced (Chance *et al.*, 1979),

but single electron reductions can occur (Fig 1.1) from electron leakage by NADH dehydrogenase (EC 1.6.5.3) or ubisemiquinone of the mitochondrial electron transport chain (McCord and Turrens, 1994). A single electron reduction of $^3O_2$ produces a free radical called superoxide anion ($O_2^{\bullet -}$, the superscript dot represents the unpaired electron). Superoxide can dismutate to form the more stable H_2O_2 (Apel and Hirt, 2004). Transition elements can donate electrons to H_2O_2 splitting the molecule into a hydroxyl anion and hydroxyl radical ($^{\bullet}OH$). The $^{\bullet}OH$ radical is the most reactive of all ROS. Hence, careful regulation of the co-occurrence of transition elements and H_2O_2 is required by plants (Fourcroy, 1999; Halliwell, 2006). There are many other ROS, including hydroperoxides associated with lipid peroxidation and the non-radical singlet oxygen ($^1O_2$) that is notably derived from stress-related photosynthesis. There are also several reactive nitrogen and reactive sulphur species (Halliwell, 2006).

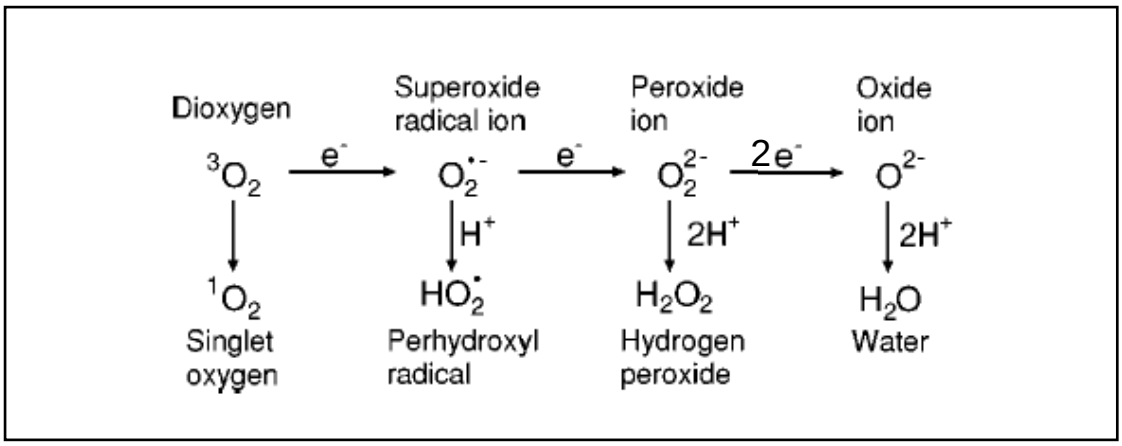


Figure 1.1 Partial electron (e^-) reductions of $^3O_2$, showing the different redox states of ROS. During respiration, 99 % of molecular O_2 is reduced by the addition of four electrons, forming H_2O. However, even under "normal" conditions, 1-2 % is still partially reduced by the transfer of only one, two or three electrons onto $^3O_2$. The production of ROS increases under stress due to imbalances in the electron transport chains (figure modified from Apel & Hirt, 2004).

1.2 Oxidative stress

Under stress respiration can become unbalanced, increasing the production of ROS from incomplete reductions of $^3O_2$ (Inze and Van Montagu, 1995; del Rio *et al.*, 2002). Photosynthesis can also be a major source of ROS especially

during stress. Oxygen molecules can be directly reduced to $O_2^{\bullet -}$ by the photosystems (Park *et al.*, 1996), which is called the Mehler reaction. The production of $O_2^{\bullet -}$ is not disastrous. It can be dealt with by enzymatic antioxidants (section 1.4) and allows electron flow when NADP concentrations are limited. If unabated, the enhanced production of $O_2^{\bullet -}$, H_2O_2 (Polle, 2001) and potentially $^{\bullet}OH$, can have detrimental consequences termed oxidative stress (Ries, 1991). The $^{\bullet}OH$ radical can react with any protein and has been shown to cause mutagenic legions in nucleic acids (Evans *et al.*, 2004). Due to its lipophilicity and redox potential perhydroxyl radicals ($HO_2^{\bullet}$), protonated forms of $O_2^{\bullet -}$, can abstract a hydrogen atom from polyunsaturated fatty acids (PUFA) of biological membranes (McCord, 2008). The single electron oxidation of PUFA creates a radical that rapidly bonds to $^3O_2$. This creates a lipid dioxyl radical, which can abstract a hydrogen atom from other PUFA, initiating a chain reaction that tears through the membrane (Halliwell and Gutteridge, 1999). Lipid peroxides are the most frequent end products, which decrease the fluidity and increase the permeability of membranes, affecting their functionality (Senaratna *et al.*, 1987; Sattler *et al.*, 2004). Typically, one $HO_2^{\bullet}$ will lead to the peroxidation of 15 PUFA (McCord, 2008). The damage of membranes is indicated by membrane permeability, which can be measured by the conductivity of the water that the material has been placed in (ISTA, 1995). Although rather crude, conductivity measurements of the incubation solution have been used as a viability indicator (Reisdorph and Koster, 1999; Leprince *et al.*, 2000; Goel *et al.*, 2003). As the production of ROS is unavoidable, aerobic organisms have evolved mechanisms that prevent oxidative stress and keep the cellular environment at a reduced redox state. Antioxidants can be non-enzymatic (section 1.3), such as ascorbic acid, or enzymatic (section 1.4), such as ascorbate peroxidase.

1.3 Non-enzymatic antioxidants

1.3.1 Low-molecular-weight thiols and the cellular redox state

The antioxidant property of low-molecular-weight (LMW) thiols is based on the sulphydryl group of their cysteine moieties. Although cysteine is an essential building block that contributes to the structure or activity of many proteins, the free amino acid is toxic at high concentrations. Therefore, plants convert cysteine into glutathione, which is the major sink of reduced sulphur in plants (Rennenberg and Brunold, 1994). Reduced glutathione (GSH) is a tripeptide (γ-glutamyl-cysteinyl-glycine) and the most abundant LMW thiol of plants (Noctor *et al.*, 2002). The sulphhydryl or thiol group (as indicated by the –SH) of GSH can donate an electron to free radicals. Due to its abundance and negative redox potential, GSH strongly contributes to the "intracellular redox environment" (Schaffer and Beutner, 2001), allowing cells to maintain a healthy reduced redox homeostasis (Noctor *et al.*, 2002). Moreover, knowing the cellular concentrations of GSH and GSSG enables the glutathione half cell reduction potential ($E_{GSSG/2GSH}$) to be calculated (Schaffer and Beutner, 2001), providing a reliable cell viability marker (Kranner *et al.*, 2006). Reduced glutathione is oxidised to a glutathiyl anion radical ($GS^{\bullet}$). Two $GS^{\bullet}$ can spontaneously bond to form glutathione disulphide (GSSG), which can be recycled back to GSH by glutathione reductase (EC 1.8.1.7) in the presence of $NADPH/H^{+}$ (Foyer and Halliwell, 1976). Glutathione disulphide is able to form mixed disulphides with thiol-containing proteins. In this way, protein-bound glutathione protects the protein thiol groups from auto-oxidation to sulphonic acids (Kranner and Grill, 1996).

1.3.2 Ascorbic acid

Ascorbic acid (AA) is able to accept electrons from free radicals, such as $^{\bullet}OH$, which creates less reactive radicle intermediates and allowing the ROS to be converted to H_2O. The oxidation of ascorbate forms monodehydroascorbate (MDHA), which rapidly disproportionates with another MDHA molecule to dehydroascorbate (DHA) plus ascorbate. The DHA and MDHA are recycled

back to AA by glutathione-dependent dehydroascorbate reductase and monodehydroascorbate reductase (Foyer and Halliwell, 1976; Smirnoff & Wheeler, 2000). Ascorbic acid is highly abundant in chloroplasts where it regulates the levels of ROS produced by photosytems.

1.4 Antioxidant enzymes

Enzymes that catalytically remove ROS include catalase, superoxide dismutase (SOD) and peroxidases (Halliwell and Gutteridge, 1999). Hydrogen peroxide is less reactive then $O_2^{\bullet -}$, hence even in low concentrations $O_2^{\bullet -}$ is converted rapidly to H_2O_2 by SOD (EC 1.15.1.1), as shown in reaction (1). The enzyme has several forms containing either copper, zinc ($Cu^{+/+2}$, $Zn^{+/+2}$), manganese or iron ($Fe^{+2/+3}$, $Mn^{+2/+3}$) in its active centre, which facilitate electron transfer from $O_2^{\bullet -}$ to H^+ ions (McCord and Fridovich, 1988).

$$(1)\quad 2O_2^{\bullet -} + 2H^+ \rightarrow {}^3O_2 + H_2O_2$$

Peroxidases (POX) form a large group of enzymes that transfer electrons from reductants to H_2O_2 forming two molecules of H_2O. In the POX cycle H_2O_2 oxidises the native form of POX (Fe^{+3}) with 2 electrons to POX-compound I^{+5}, which can then be reduced back to the native form by by two single electron reductions via POX-compound II^{+4}. Peroxidases are split into three superfamilies. The first is animal POX, which includes glutathione POX also found in plants, the second is catalase and the third POX is found in plants, bacteria, yeast and fungi. Catalase (EC 1.11.1.6) is able to rapidly break down high concentrations of H_2O_2 as shown in reaction (2).

$$(2)\quad 2H_2O_2 \rightarrow 2H_2O + {}^3O_2$$

Electron transfer by catalase occurs through its porphyrin ring of four haem groups and like SOD, uses no co-substrates. This reaction is feasible because H_2O_2 can be a weak reductant as well as a strong oxidant.

In the third POX superfamily there are three further classes. Class I are intracellular, class II are only found in fungi and class III are extracellular and

specific to plants (Hiraga *et al*., 2001). Ascorbate POX (EC 1.11.1.11), a class I POX, is very efficient at removing H_2O_2 in the presence of AA (Asada, 1992). Glutathione POX (EC 1.11.1.9) uses GSH as an electron donor, but in plants unlike animals, is induced rather than being part of the constitutive stress response (Foyer and Noctor, 2000). Glutathione POX is inefficient at removing H_2O_2, but efficiently decontaminates alkyl and lipid peroxides (Eshdat *et al*., 1997). Extracellular class III POX (EC 1.11.17) can typically metabolise many electron donors in the presence of H_2O_2 (Hiraga *et al*., 2001), although reaction rates vary (Wallace and Fry, 1999). Seventy three extracellular POX (ECPOX) genes have been identified in the genome of *Arabidopsis thaliana* L. Heynh, 23 of which were translated in non-stressed plants, suggesting that many genes are either redundant or specifically induced (Tognolli *et al*., 2002). Although not yet fully classified, it is expected that ECPOX are functionally specialised because of their many divergent promoters and highly variable regions of protein sequences (Cosio and Dunand, 2009). So far, several ECPOX functions have been characterised, including lignification (Blee *et al*., 2001; Ros-Barceló *et al*., 2004), the oxidative coupling of cell wall proteins following wounding or pathogen challenges (Bradely *et al*., 1992; Otte and Barz, 1995), breaking down H_2O_2 using catalase-like activity (Hiner *et al*., 2001; Mika *et al*., 2004) and the production of ROS, such as H_2O_2 (Pichorner *et al*., 1992; Bolwell *et al*., 1995), $O_2^{\bullet-}$ (Hallilwell, 1978; Kawano, 2003) and $^{\bullet}OH$ (Chen and Schopfer, 1999; Liszkay *et al*., 2004). Thus, many enzymes from the three POX superfamilies are associated with diverse stress responses, including a role for ECPOX in the production, as well as breakdown, of ROS.

1.5 Reactive oxygen species as signalling molecules

Despite the harmful and damaging nature of ROS described so far, ROS are also signalling molecules produced in response to stress (Alvarez *et al*., 1998; Neill *et al*., 2002; Laloi *et al*., 2004; Rhee, 2006). Moreover, the presence of ROS is necessary for a number of normal 'housekeeping' operations. For example, $O_2^{\bullet-}$ is essential for growth (Foreman *et al*., 2003), cellular

organisation (Gapper and Dolan, 2006) and gravitropism (Joo *et al*., 2001) of plant roots. Enzymes that produce ROS are often found within or have their activity directed towards the apoplast (Allan and Fluhr, 1997; Bolwell *et al*., 2002). The production of ROS in the apoplast in contrast to the reduced cellular environment creates a trans-membrane redox gradient between the apoplast and cytosol (Antunes and Cadenas, 2000; Stone, 2004). Hydrogen peroxide can diffuse through membrane channels or aquaporins into the cytoplasm (Chance *et al*., 1979; Henzle and Steudle, 2000; Bienert *et al*., 2007), oxidise receptors and induce defence strategies. For example, in tomato leaves the transcription of defence-related genes is influenced by H_2O_2 and without its production in response to stress no gene up-regulation occurs (Orozco-Cárdenas *et al*., 2001).

Although stress responses are not fully understood at a molecular level, research undertaken in non-plant organisms has revealed some insights. The maintenance of a reduced cellular state facilitates the contrast and effect of ROS in signal transduction (Fedoroff, 2006; Rhee, 2006). ROS can target thiol receptor proteins (Apel and Hirt, 2004; Foyer & Noctor, 2005), which are oxidised switching their activity. Specific redoxins can then reinduce the receptor function by re-establishing the reduced state (Wood *et al*., 2004). A bacterial protein called oxyR has been identified that controls the transcription of antioxidant proteins (Storz *et al*., 1990). The oxyR activity is up-regulated after oxidation of its two cysteine moieties (Lee *et al*., 2004), becoming deactivated by glutaredoxin (Zheng *et al*., 1998). Also, the transcription of many stress-related genes, including a thioredoxin for a self-regulatory mechanism, is controlled by the oxidation of cysteine moieties within yAP-1 protein of yeast (Delaunay *et al*., 2002; Wood *et al*., 2004).

Redox sensitive proteins found in plants include mitogen activated protein kinases (MAPK) (Kovtun *et al*., 2000; Samuel and Ellis, 2002). Kinases are regulatory enzymes that modify protein conformation by phosphorylation, affecting target receptor or enzyme activity. Often, they operate in a three tier cascade. The terminal kinase responsible for functional modification is

activated by a different kinase (MAPKK), which itself is activated by an upstream kinase (MAPKKK). The stress signalling network is complex, intertwining with other sensory channels and interacts with growth regulators, with distinct stresses often activating the same kinases (Desikan *et al.*, 2001; Apel and Hirt, 2004). Similarly, the protection afforded by one stress can share overlapping features to protect against other stresses, known as 'cross-tolerance' (Bowler and Fluhr, 2000).

The rapid production of extracellular ROS, termed the oxidative burst, is a universal plant response following the sensing of a whole range of abiotic and biotic stresses, including pathogen challenges (Doke, 1983; Thordal-Christensen, 1997; Bolwell *et al.*, 2002). A second oxidative burst can occur several hours after pathogen recognition (Baker and Orlandi, 1995; Levine *et al.*, 1996), which can induce apoptosis of infected cells in an attempt to contain the pathogen (Jabs *et al.*, 1996; Lamb and Dixon, 1997). Wounding also stimulates an oxidative burst (Roach *et al.*, 2008; Minibayeva *et al.*, 2009), possibly induced by fractured oligosaccharide fragments (Legendre *et al.*, 1993; Côte and Hahn, 1994). The induced production of ROS may be part of signalling networks, as discussed, or may also be used in many physiological processes, which is described next.

1.6 Physiological roles of extracellular reactive oxygen species

The apoplast of plants has received considerable attention for the location of ROS because many enzymes that produce or metabolise ROS are either found in the extracellular matrix or have their activity directed towards it (Doke, 1985; Allan and Fluhr, 1997; Bolwell *et al.*, 2002). In fact, several reports have suggested that high concentrations of extracellular H_2O_2 may reach antimicrobial levels directly combating pathogens (*citations in* Murphy *et al.*, 1998). Following pathogen-induced elicitation (Otte and Barz, 1996) or wounding (Bradley *et al.*, 1992) rapid H_2O_2-dependent cross-linking of cell wall proteins occurs, reinforcing the cell walls. This shares similarities to lignification, as it is mediated by ECPOX that requires H_2O_2 and it strengthens

the cell walls (Fry, 1986; Ros-Barceló *et al*., 2004). Conversely, H_2O_2 may also be involved in loosening cell walls, thereby allowing cellular extension. Plant growth requires breakages of the existing primary cell wall polysaccharide matrix, which can be cleaved by $^{\bullet}OH$ that maybe produced by ECPOX in the presence of H_2O_2 (Chen and Schopfer, 1999; Liszkay *et al*., 2004; Müller *et al*., 2009). Hence, extracellular ROS play many important roles. However, due to their potentially destructive nature, their production in precise locations is under careful control, which can be provided by specific enzymes.

1.7 Enzymatic production of reactive oxygen species

1.7.1 Membrane bound NAD(P)H oxidases

Much research on enzymatic production of ROS has focused on the membrane bound NAD(P)H oxidases (NOX) of plants, analogous to the enzymes characterised in mammalian phagocytes. The enzyme has been associated to the rapid production of $O_2^{\bullet -}$ in response to plant pathogen challenges (Doke, 1983; Lamb, 1994; Torres *et al*., 2005) and plant wounding (Cona *et al*., 2006). The membrane-bound complex transfers an electron, using its flavin-containing active centre, from NAD(P)H in the cytosol onto $^3O_2$ and releasing $O_2^{\bullet -}$ into the apoplast. However, due to the complications of extracting intact membrane-bound proteins, there has been limited characterisation of NOX. As Schopfer *et al*., (2008) state "there are obviously several [plasma membrane] enzymes with overlapping substrate specificities that have not yet been properly disentangled". However, ECPOX have also been identified in pathogen-induced ROS production (Bolwell *et al*., 1995) and the relative contribution of each enzyme has been fiercely debated in the literature (Torres *et al*., 2005; Bindschedler *et al*., 2006).

1.7.2 Extracellular peroxidases

Early studies showed that ECPOX were capable of producing ROS *in-vitro* (Halliwell, 1978; Pichorner *et al*., 1992). However it was not until 1995 that Bolwell *et al*., (1995) published that the reaction occurred *in-vivo*, using plant

cell cultures elicited with pathogen cell wall fragments. Central to the process was an alkalisation of the apoplast, but it was also noticed that an apoplastic calcium efflux occurred. Since then, many papers on ECPOX-derived ROS production have been published (e.g. Kawano and Muto, 2000; Liszkay *et al.*, 2004; Bindschedler *et al.*, 2006; Rouet *et al.*, 2006; Minibayeva *et al.*, 2009). The production of ROS by ECPOX has been associated with alleviating seed dormancy (Schopfer *et al.*, 2001), possibly the first ROS-producing function described in seeds. The pathway of ECPOX-mediated $O_2^{\bullet-}$ production suggests the requirement of an apoplastic reductant, which is yet to be fully confirmed. For ECPOX to produce $O_2^{\bullet-}$ H_2O_2 is also required (Halliwell, 1978; Hiner *et al.*, 2001), which can be provided by other enzymes, such as amine oxidases.

1.7.3 Amine oxidases

Amine oxidases (AOX) are enzymes that produce H_2O_2 by breaking down (poly)amines (Padiglia *et al.*, 1991). Two classes of AOX have been discovered, which contain either copper or flavin in their active centre (Medda *et al.*, 1995). Copper-containing AOX are abundantly found in the apoplast of legumes, such as *P. sativum* (Federico and Angelini, 1986; Angelini *et al.*, 1990) and produce H_2O_2 most effeciently in the presence of putrescine (Pientrangeli *et al.*, 2007), as shown in reaction (3).

(3) Putrescine + O_2 + H_2O $\rightarrow$ 1-pyrroline + NH_3 + H_2O_2

In *P. sativum* seedlings, AOX have been shown to be confined to cortical cells, the xylem and at cell junctions close to the meristem (Laurenzi *et al.*, 2001). Hydrogen peroxide production from AOX activity occurs rapidly following elicitation (Allan and Fluhr, 1997) and wounding (Angelini *et al.*, 2008). Furthermore, it has been shown that an up-regulation of genes involved with polyamine biosynthesis occurs in response to distinct abiotic stresses (Urano *et al.*, 2003), suggesting that polyamines are integral to many plant stress responses. The accumulation of polyamines can not only lead to the production of H_2O_2, but are also linked to the synthesis of nitric oxide

(Tun *et al.*, 2006), a molecule that mediates other responses to stress, such as cell death during pathogen infections (Delledonne *et al.*, 1998; Durner *et al.*, 1998).

1.7.4 Lipoxygenases

Other ROS-producing enzymes particularly abundant in leguminous seeds (Loiseau *et al.*, 2001) are lipoxygenases (LOX) that catalyse the dioxygenation of PUFA. As shown in reaction (4), the major reaction products are fatty acid hydroperoxides, but LOX-mediated formation of $^1O_2$ and $O_2^{\bullet -}$ has also been observed (Kanofsky and Axelrod, 1986; Zuo *et al.*, 2004).

(4) Fatty acid + $O_2 \rightarrow$ fatty acid hydroperoxide

Following germination, major increases in lipoxygenase (LOX) activity have been associated with the onset of epicotyl elongation (Bailly *et al.*, 2002), in accord with suggestions that LOX-mediated membrane reorganisation is critical to cell growth (Maccarrone *et al.*, 1994; Porta *et al.*, 1999). Also, the participation of LOX in the wounding response is of particular interest because jasmonates involved in downstream signalling can be produced from the products of LOX metabolism.

In summary, there are several enzymes capable of producing ROS in plants. However, their occurrence and roles in seeds and seedlings has been sparsely reported. The introduction now switches focus to seeds, the model systems used in this thesis, providing some background on their physiology.

1.8 Seeds

Seeds can be classified by their tolerance to desiccation. Seeds that tolerate desiccation are called "orthodox" and seeds that do not tolerate desiccation are called "recalcitrant" (Roberts, 1973). An estimated 90 percent of seeds are orthodox (Dickie and Pritchard, 2002). Some seeds, such as *Coffea arabica* L., are less easily classified by their desiccation or temperature sensitivity and hence are termed "intermediate" (Ellis *et al.*, 1990; Dussert *et al.*, 1999).

During the final stage of development, orthodox seeds undergo maturation drying as part of normal seed development, a process absent in recalcitrant seeds (Hong and Ellis, 1990; Kermode and Finch-Savage, 2002). Therefore, it has been suggested that recalcitrant seeds are developmentally immature (Finch-Savage, 1992). Water content (WC) and temperature affect seed longevity (Ellis and Roberts, 1980; Dickie *et al*., 1990) because metabolic processes influence seed deterioration in a hydrated state. Respiration in orthodox seeds is down-regulated before desiccation, becoming undetectable below a WC of around 20 %, on a fresh weight (FW) basis (Vertucci, 1989). In desiccated orthodox seeds the cytoplasm, although still fluid, behaves as a solid and is called a glass (Bewley and Black, 1994). The glassy state restricts molecular mobility, although seed ageing can still occur through oxidative reactions (Bailly *et al*., 2004). In recalcitrant seeds desiccation damages cellular structures ill-prepared for the absence of water, which is termed desiccation damage *sensu stricto* (Pammenter and Berjak, 1999). Organelle integrity is lost at a relatively high WC (Levitt, 1980), while macromolecular damage occurs towards full desiccation (Wolfe and Bryant, 1999). However, often viability is lost at higher WCs and the amount of water loss recalcitrant seeds tolerate varies between species (Sun *et al*., 1996) and can also be affected by desiccation rates (Pammenter *et al*., 1991; Berjak *et al*., 1993). For example, recalcitrant tea (*Camellia sinensis* L. Kuntze) embryonic axes lose viability at a WC of 41 % FW when desiccated slowly, whereas when desiccated much faster they can survive drying to a WC of 29 % FW (Berjak *et al*., 1993). Partially desiccated embryonic axes of some species will tolerate long-term storage in liquid nitrogen, as used for the cryopreservation of recalcitrant germplasm (Corredoira *et al*., 2004).

The effect of desiccation rate upon recalcitrant seed viability highlights the influence of metabolism on desiccation-induced recalcitrant seed deterioration. During water loss, respiration becomes unbalanced leading to incomplete reductions of $^3O_2$ (section 1.1) and enhanced production of ROS (Hendry, 1993; Finch-Savage *et al*., 1994; Cŏme and Corbineau, 1996;

Kranner *et al*., 2006). Antioxidants in seeds that combat ROS production include AA and GSH (section 1.3). Ascorbic acid is present during seed development, being initially transferred from the maternal plant to the seed (Tommasi *et al*., 1999). Seeds of broad bean (*Vicia faba* L.) gain capabilities to synthesise AA 30 d after anthesis just before the seed desiccates (Arrigoni *et al*, 1992). Due to its instability, AA is not stored in desiccated tissue (Tullio and Arrigoni, 2003), but the synthesis of AA is crucial for germination when it is also required for cell cycle activity (Pallanca and Smirnoff, 1999). In contrast, recalcitrant seeds maintain high levels of AA from maturation to germination (Tommasi *et al*, 1999). Unlike AA, GSH is stable in the desiccated state and highly abundant in orthodox and recalcitrant seeds (Kranner *et al*., 2006). Interestingly, seeds contain more glutathione, on a dry weight basis, than other tissues of the plant (Klapheck, 1988). This is perhaps related to the protection afforded by glutathione in the desiccated state. This may be particularly relevant to orthodox seeds where GSSG levels rise during desiccation (Cairns *et al*., 2006).

In this thesis, the recalcitrant seeds of *C. sativa* were used to enhance our knowledge of the production of ROS in response to stress. The species has ecological and economic value. The trees are important components of European forests, being commercially exploited for wood and the seeds are edible with an annual worldwide harvest of 1,200,000 tonnes (FAOSTAT, 2006). The orthodox seeds of *P. sativum* are used to characterise the production of ROS during germination and in response to stress. Organ development in plants is typically postembryonic, allowing a unique opportunity to study the production and influence of ROS during seedling development. The apical end of the embryonic axis contains the shoot meristem, producing above-ground organs namely the stems, the leaves and the flowers. The basal end of the embryonic axis produces the radicle and eventually the whole root system.

1.9 Aims

In the field of plant science, the beneficial aspects of ROS in signalling have been receiving considerable interest (Foyer and Noctor, 2009), but the significance to seeds and seedlings is less well characterised. For example, although it is well known that plant leaves or roots produce an oxidative burst in response to wounding (Orozco-Cardenas *et al*., 2001; Minibayeva *et al*., 2009), no studies have shown that seeds behave similarly. The current understanding of seed enzymes that metabolise ROS is almost entirely restricted to antioxidant processes, although there are some exceptions (e.g. Schopfer *et al*., 2001). The seeds and seedlings of *P. sativum* and *C. sativa* were used to characterise the production of ROS in response to various stresses that could be induced in a controlled manner.

In chapter 3 glutathione measurements were made in embryonic axes and cotyledons from variably desiccated *C. sativa* seeds. This provided an indication of oxidative stress during desiccation-induced viability loss. Glutathione is one of several LMW thiols and consideration is also given to cyst(e)ine, γ-glutamyl-cyst(e)ine and cyst(e)inyl-glycine, which may all contribute to the cellular redox homeostasis (Jones et al., 2000). The production of ROS by *C. sativa* embryonic axes in response to wounding and desiccation was investigated, which is particularly relevant to the conservation of these seeds because embryonic axis excision and desiccation is routinely used in cryopreservation protocols (Corredoira *et al*., 2004).

Hardly any information is available on the enzymes involved in ROS production in seeds. Building on the results of chapter 3, ECPOX-mediated ROS production by *C. sativa* embryonic axes was characterised in chapter 4. The production of ROS may well be related to signalling (Laloi *et al*., 2004), but it is also known that ROS participate in physiological processes, such as cell wall modification (Passardi *et al*., 2004, Liszkay *et al*., 2004). Thus, it was hypothesised that ROS may ameliorate desiccation stress in certain time windows. The application of H_2O_2 to partially desiccated *C. sativa* seeds and

P. sativum seedlings was investigated in relation to germination, growth and the establishment of secondary root meristems in chapters 4 and 5.

The production of ROS during germination of aged and non-aged *P. sativum* seeds was investigated in chapter 5. Following germination an orthodox seed forgoes its desiccation tolerance. The production of ROS as a consequence of desiccation was explored in *P. sativum* seedlings in chapter 5. Extracellular POX have been associated with the elicitor-induced (Bindschedler *et al.*, 2006) and the wounding-induced (Minibayeva *et al.*, 2009) oxidative burst, but the reductant(s) remain elusive. In chapter 6, the participation of extracellular enzymes in wound response of *P. sativum* seedlings was explored and attempts were made to identify potential enzyme substrates as well as to characterise the precise nature of the enzymes involved.

1.10 The project and participation of other collaborators

This PhD was mainly funded through a research project grant by the Leverhulme trust awarded to Dr Ilse Kranner of the Seed Research Department (SCD) of the Royal Botanic Gardens (RBG) Kew, UK. Other financial support for this work, through using the facilities of the SCD, came from the Millennium Commission, the Wellcome Trust and Orange Plc. The Royal Botanic Gardens, Kew is supported grant-in-aid from Defra. Collaborators included Prof. Richard Beckett and Miss Mariyana Ivanova of the University Kwa-Zulu Natal, South Africa, Dr. Farida Minibayeva of the National Academy of Sciences, Kazan, Russia, Dr. Louise Colville and Mr Ian Green of the SCD, RBG Kew, UK. Their contribution to the research, which is shown in four figures and two tables, has been accredited in chapter 2 and figure and table legends.

Chapter 2

Materials and Methods

The materials and methods for all four research chapters are provided here. Many assays have been used for more than one chapter and to prevent repetition, each assay has been described only once. Any modifications in the desiccation times or assay volumes to account for different material sizes are included in figure legends as are the number of samples per replicate and replicates per treatment.

2.1 Chemicals

Analytical grade chemicals were purchased from Sigma (St. Louis, MO, USA), Fisher (Loughborough, Leicestershire, UK) or Fluka (Buchs, Switzerland) and all solutions were made up with distilled deionised water (dH_2O) unless indicated otherwise.

2.2 Seeds

Castanea sativa Mill. seeds were obtained from the from Corpo Forestale dello Stato Centro Nazionale per lo Studio e la Conservazione della Biodiversità Forestale, Peri-Vr Italy, in autumn 2006 and 2007 and kept at 5 ^{o}C for up to 12 weeks in a plastic bag until used. Experiments below are labelled regarding years seeds were used. Seed infection rates increased with storage, starting at *c.* 10 % on arrival and increasing to *c.* 60 % after 12 weeks. Only non-infected seeds were used in the experiments. Tissue used for biochemical assessments (LMW thiols and enzyme extraction) were plunged into liquid nitrogen, freeze dried for 48 h and stored at -70 ^{o}C sealed with silica gel until used.

Pisum sativum L. cv. Rondo (organically certified, Soil Association, UK) were purchased from Kings Seeds (Colchester, Essex, UK) and stored at 15 ^{o}C and 15 % relative humidity (RH) at an equilibrium water content (WC) of 6.6 %

fresh weight (FW). Damaged or dirty seeds were removed before all experiments.

2.3 Desiccation and measurement of water content

Castanea sativa seeds were loosely spread upon wire racks allowing air space between all seeds and desiccated at 15 % RH and 15 ^{o}C for up to 21 d. The pericarp was removed prior to desiccation. Isolated embryonic axes were desiccated in stream of air (*c.* 0.45 m s^{-1}) at 20 ^{o}C and *c.* 60 % RH in a flow bench for up to 8 h. *Pisum sativum* seedlings were desiccated over silica gel at 3 % RH and 20 ^{O}C for up to 24 h. To prevent pre-experimental desiccation, isolated embryonic axes were placed in a Petri dish in the top half of a sealed 1 litre Le Parfait Jar (VMC, France) that was half filled with H_2O until sufficient embryonic axes had been excised.

Dry weight (DW) of seeds, embryonic axes and seedling axes was determined gravimetrically after fully drying at 103 ºC for 17 h. Throughout this thesis WC is expressed on a FW basis expressing the percent of H_2O of total mass and calculated by:

$$WC = (FW - DW) / FW \times 100$$

2.4 Viability assessments

All seeds and embryonic axes were placed at 25 ^{o}C under an 8 h day (warm white fluorescent light at a photon flux density of 15 μmol m^{-2} s^{-1}) / 16 h night cycle unless stated otherwise.

2.4.1 *Castanea sativa*

Seeds were germinated by placing their flat side in contact with 0.8 % (w/v) water agar, pH 6.5. This allowed direct water uptake from the agar into the cotyledon that surrounded the invested embryonic axis. Successful germination was defined as radicle emergence by at least 10 mm, and scored regularly until all seeds had either germinated or become heavily infected and started disintegrating. In 2006, seeds were equally spread upon 1 cm depth

of agar, with 20 seeds per 6 x 12 x 17 cm lidded transparent box. In 2007, individual seeds were placed in 40 mm diameter plastic pots each containing 20 ml of agar to prevent cross contamination between seeds, with 8 seeds in pots in each box. Five replicates (boxes) were used for all germination tests.

Embryonic axes were isolated from *Castanea sativa* seeds by slicing through the cotyledon attachments, taking care not to damage the shoot meristem. Embryonic axes were either excised from seeds and then desiccated or isolated from desiccated seeds, as described below. When using excised embryonic axes for desiccation, the axes were placed in a sealed humidity chamber, as described above in section 2.3, immediately after excision, until a sufficient number had been obtained for each replicate. The viability of embryonic axes desiccated after isolation was conducted in 2006 by Mr Ian Green (SCD, RBG Kew, UK). In 2006 embryonic axes were not sterilised before culturing. Embryonic axes from pre-desiccated seeds used for viability measurements in 2007 were sterilised in 50 mM sodium dichloroisocyanurate for 10 min, rinsed 3 times and then soaked for 1 h in H_2O. Embryonic axes from 2006 and 2007 were cultured on McCown's Woody Plant Basal Salt Mixture (Sigma) with 50 mg l^{-1} myo-inositol, 0.25 mg l^{-1} thiamine, pyridoxine and pantothenerate added (Norstog, 1973), 30 g l^{-1} sucrose and 0.8 % (w/v) agar, pH 5.6. A separate 2 cm^3 chamber was provided for each axis. Cultured embryonic axes were kept in the dark for the first 7 d, then moved into the light, as above, and transferred to new 9 cm diameter Petri dishes after 10 d. Viability was measured by root or shoot elongation in aseptic tissue culture by greater than 5mm rather than callus formation alone.

To test the effect of H_2O_2 on the germination of non-desiccated chestnuts, seeds were soaked in 0, 0.01, 0.50 or 1.00 M H_2O_2 for 1 h. One M H_2O_2 was used to study the effects of H_2O_2 treatment on the germination of desiccated seeds. Following desiccation, seeds were treated by submerging for 1 h in H_2O or 1 M H_2O_2 and germinated as above.

The effect of incubating *C. sativa* embryonic axes in H_2O_2 or GSH on axis vigour and viability was also measured. When treating seeds, the embryonic axis is protected from the incubation solution. Hence, when treating embryonic axes, lower H_2O_2 concentrations were used. Embryonic axes were isolated from seeds desiccated for 5 d. After sterilisation in 50 mM sodium dichloroisocyanurate for 10 min, embryonic axes were rinsed 3 times and then submerged for 1 h, sufficient time to for the axis to fully rehydrate, in solutions containing 0.1, 1.0 or 10.0 mM H_2O_2 or the same concentrations of GSH and cultured as above. The best growth rates were achieved with 0.1 mM H_2O_2, with no positive effect observed for GSH, and 0.1 mM H_2O_2 was used to study the effects on embryonic axes excised from 10 d and 21 d desiccated seeds in comparison with H_2O treatment.

2.4.2 *Pisum sativum*

Seeds were rinsed in pure ethanol, sterilised in 4 % hypochlorite (Domestos, Unilever, UK) for 5 min, washed in dH_2O until chlorine odour was absent and then soaked in dH_2O for 1 h. Soaking in dH_2O for 1 h increased initial imbibition rates and promoted total germination of non-aged seeds, which occurred within 56 h. *Pisum sativum* seeds were placed with their hilum in contact with moist germination paper (Fisherbrand, Fisher, UK). A thin layer of damp paper towel was placed over the seeds, which were germinated at 25 ^{o}C. Germination was defined as radicle elongation after testa rupture by at least 2 mm.

Seeds were artificially aged at 45 ^{o}C and 72 % RH, corresponding to seed WC of 12 % FW, for up to 102 d, sufficient time for total viability to be lost. Before ageing, seeds were pre-equilibrated to 12 % WC at 81 % RH and 20 ^{o}C using a 3.8 M LiCl solution. Ageing was conducted in sealed jars (n = 5 replicates of 200 seeds jar^{-1} for each ageing interval) with seeds suspended on plastic mesh above a solution of 5.2 M LiCl (72 % RH).

The effect of desiccation on the viability of *P. sativum* seedlings at various developmental stages was measured. Seedling axes were isolated

from 56 h imbibed (fully germinated) seeds, referred to as seedling axes, and separated into groups of similar radicle lengths, as indicated in figures or figure legends. After desiccation over silica gel, seedling axes were sterilised in 50 mM sodium dichloroisocyanurate for 30 min, rinsed three times and cultured as above.

The effects of a 30 min treatment of 10 mM H_2O_2 on root and shoot growth of *P. sativum* seedling axes desiccated for 2 h over silica gel was also measured. Seedling axes were separated into groups of similar radicle lengths, desiccated, submerged in either 10.0 mM H_2O_2 or H_2O for 30 min and then sterilised in 50 mM sodium dichloroisocyanurate for 30 min. Seedling axes were then rinsed 3 times and placed on half strength Murashige and Skoog (MS) media (Sigma, USA), pH 5.6, and incubated at 25 ^{o}C and viability was measured after 21 d of culturing. Root viability was counted as new secondary root growth (after even the slightest desiccation the radicle meristem always died), and shoot viability was counted when more than 5 mm of shoot growth was observed during 21 d culturing.

2.4.3 Assessment of membrane permeability

Membrane permeability was assessed by the electrolyte leakage in the imbibition solution into which the seed material had been placed in, referred to as the leachate, using a CM100 conductivity meter (Reid, Durban, SA). Conductivity measurements of the leachate (μS g DW^{-1}) were taken in a temperature-controlled room at 20 ºC after submerging *C. sativa* embryonic axes or *P. sativum* seeds in 1.6 ml and 3.0 ml of dH_2O, respectively. Data were recorded every 30 min for up to 12 h. Leachates and seeds or embryonic axes from each well were dried at 103 ºC for 17 h to determine DW. The electrolyte leakage from desiccated or non-desiccated *C. sativa* embryonic axes was measured in 2006. After excision, embryonic axes were either placed in a humidity chamber, as described above in section 2.3, or in a stream of air (*c.* 0.45 m s^{-1}) of a flow bench. After 6 h, the WC of embryonic axes in the humidity chamber was 66 ± 3 % and the WC of embryonic axes

desiccated in the flow bench was 26 ± 3 %. The electrolyte leakage from *P. sativum* seeds aged for 0, 23 and 102 d was also measured, as above.

2.4.4 Determination of glutathione, glutathione disulphide and glutathione half-cell reduction potential of *Castanea sativa*

Low-molecular-weight thiols and disulphides were measured according to Kranner (1998). Tissue was extracted from seeds, 2007, desiccated for 0, 5, 10 and 21 d. Five replicates each consisting of 5 embryonic axes (*c.* 28 mg DW) or 5 cotyledon pieces from individual seeds (*c.* 35 mg) were used for analysis. Freeze-dried material was ground to a fine powder in a hermetically closed, liquid nitrogen-cooled Teflon vessel using a Retsch MM200 laboratory mill. The powder was stored at −70 °C in humidity-proof vials until use. Ground samples were extracted by vortexing for 10 s in 1 ml of ice cold 0.1 M HCl and centrifugation at 12,000 *g* for 40 min. 120 µl and 400 µl of the supernatant were used for further treatments to measure disulphides and total LMW thiols plus disulphides, respectively. The pH of extracts was adjusted to between 8.0 – 8.3 by adding 1.5 times the original extract volume of 200 mM pH 9.9 CHES buffer. Total LMW thiols together with disulphides were determined after reduction of disulphides by adding 30 µl of 3 mM dithiothreitol (DTT). Thiol groups were labelled with 30 µl of 15 mM monobromobimane (MB). After 15 min the reaction was halted by adding 240 µl of 0.25 % methanesulfonic acid. Low-molecular-weight thiols were separated in 10 µl of sample using a gradient of high performance liquid chromatography (HPLC)-grade methanol to 'solvent A' (0.25 % v/v acetic acid, pH 3.85) by reversed-phase HPLC using a RP-18 column, and detected with a fluorescence detector (excitation: 380 nm, emission: 480 nm). Disulphides were measured after blocking reduced thiol groups with 30 µl of *N*-ethylmaleimide (NEM) added to 120 µl of sample, followed by removal of excess NEM with toluene (repeated five times). 120 µl of NEM-treated extract was then reduced by DTT and thiols were labelled with MB as described above. Samples were calculated by comparison to standards of GSH, cysteine,

γ-glutamyl-cysteine and cysteinyl-glycine, considering that one mole of disulphide corresponds to two moles of LMW thiols.

Half-cell reduction potentials ($E_{RSSR/2RSH}$ where R = any LMW thiol or disulphide, S = sulphur and H = hydrogen) were calculated using the Nernst equation as shown below. The equation uses the molar concentrations of thiols and disulphides, which were calculated by using the WCs measured immediately following desiccation. $E^{0'}$ is the standard redox potential of glutathione, i.e. at pH 7.0. Parentheses in cyst(e)ine indicate LMW thiol and disulphide, which were both used to calculate $E_{RSSR/2RSH}$. The $E^{0'}_{RSSR/2RSH}$ values used for glutathione, cyst(e)ine, cyst(e)inyl-glycine, and glutamyl-cyst(e)ine were -240 mV (Rost and Rapoport, 1964), -226 mV, -226 mV (Jones *et al.*, 2000) and -234 mV (Birtić S, personal communication), respectively, which were adjusted to compensate for a cytosolic pH of 7.3 by the subtraction of 18 mV; **R** is the gas constant (8.314 K^{-1} mol^{-1}); **T** is the temperature during desiccation treatments (288 K); **n** is the number of electrons transferred (2); **F** is the Faraday constant (9.6485 x 10^4 C mol^{-1}).

½ cell reaction: **2 LMW thiol → LMW disulfide + 2 H^+ + 2 e^-**

$$E^{0'} - \frac{RT}{nF} \ln \left(\frac{[\text{LMWthiol}]^2}{[\text{LMWdisulphide}]} \right)$$

2.5 Spectrophotometric measurements of extracellular reactive oxygen species

To measure the production of ROS, seeds or seedlings were incubated in a solution containing the compounds capable of reacting with ROS, and the leachates were analysed. Therefore, measurements were non-invasive and could be made on living seeds and seedlings.

2.5.1 Superoxide

Extracellular $O_2^{\bullet -}$ production was estimated by the oxidation of epinephrine to adrenochrome, which was determined colourimetrically using an extinction

coefficient of the product at A_{480} of 4020 M^{-1} cm^{-1} (Misra and Fridovich, 1972; Takeshige and Minakami, 1979).

Excised *C. sativa* embryonic axes or *P. sativum* seedlings were gently shaken in 1.5 – 5 ml of 1 mM epinephrine, adjusted to pH 7.0 or placed in 50 mM K-phosphate buffer for up to 1 h, as indicated in the figure legends. Colorimetric measurements are subject to interference from non-specific adsorption. Therefore, measurements were compensated by the subtraction of comparable values (measurements in the absence of epinephrine), as employed when using *C. sativa* embryonic axes, or after suspended material had been removed from the incubation solution by centrifugation at 13,000 *g* for 5 min, as employed when using *P. sativum* seedling axes that sometimes were accompanied by some suspended debris. The specificity of the epinephrine assay to $O_2^{\bullet -}$ was variable. When $O_2^{\bullet -}$ production is shown in the figure axis legend measurements were also taken in the presence of 200 units ml^{-1} SOD and subtracted from measurements taken in its absence. Otherwise only adrenochrome formation is shown.

2.5.2 Electron spin resonance measurements of superoxide production

Electron spin resonance (ESR), courtesy of Dr Farida Minibayeva (Russian Academy of Sciences, Kazan, Russia) was used to confirm the nature of the ROS involved in the oxidative burst following the wounding of *Castanea sativa* embryonic axes. Tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid disodium salt, Sigma) was used as a spin trap (McRae *et al*., 1982). This method measures the increase in the amplitude of the ESR spectrum caused by the formation of a semiquinone radical as a result of the reaction of Tiron with superoxide. The Tiron semiquinone radical is relatively stable and its ESR signal remains constant for at least 15 min. ESR signals were recorded at 20 ºC with a radiospectrometer RE 1306 (Smolensk, USSR) equipped with a temperature control device. Excised embryonic axes were shaken for 15 min in distilled water, then Tiron (final concentration 50 mM) was added, and

embryonic axes and solution were well mixed for 1 – 2 min. After mixing, embryonic axes were discarded, and the pH of the solution adjusted to 8.5, which was the optimum for the formation of the Tiron semiquinone radical. ESR spectra were obtained at a modulation of 100 kHz with an amplitude of 1.0 G and a time constant of 0.3 s.

2.5.3 Stimulating enzymes separated by PAGE to produce superoxide in-gel

In order to induce enzymes to produce $O_2^{\bullet -}$ following polyacrylamide gel electrophoresis (PAGE) (see section 2.8), gels were pre-equilibrated in 0.1 M phosphate buffer, pH 7.0, containing 10% glycerol and 0.5 mM $MnCl_2$ for 1 h. Thereafter, they were stained in the same buffer containing 0.5 mM nitroblue tetrazolium (NBT) with various electron donors, as described in figure legends. The appearance of purple formazan bands indicates $O_2^{\bullet -}$ production (Serrano *et al.*, 1994; López-Huertas *et al.*, 1999).

2.5.4 Hydrogen peroxide

Extracellular H_2O_2 production was determined colourimetrically using xylenol orange and an extinction coefficient of A_{560} was calculated using a calibration curve (Gay and Gebicki, 2000). 'Reagent A' comprised of 25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$ and 2.5 M H_2SO_4. 'Reagent B' contained 125 µM xylenol orange and 100 mM sorbitol, and the 'working reagent' contained 1:100, reagent A:reagent B, respectively, combined 30 min before measurements. The amount of H_2O_2 in the incubation solution, defined as solution that material had been incubated in and then removed from, was determined after combination with the working reagent and incubating for 20 min. The production of H_2O_2 by *P. sativum* seeds during germination, courtesy of Miss Mariyana Ivanova (School of Botany, Universtiy Kwa-Zulu Natal, South Africa) was taken by shaking seeds in 5 ml of distilled water, pH 7.0, and a 0.25 ml aliquot of the leachate was added to 1.5 ml of working reagent. The production of H_2O_2 by wounded *P. sativum* seedling axes was measured in 0.5 ml of incubation solution added to 3ml of working reagent. The

breakdown of 10 mM H_2O_2 by 2 h desiccated *P. sativum* seedling axes was measured in 50 µl of incubation solution diluted by 1:50. The diluted sample was added to 3 ml of working reagent. To test the specificity of the assay as shown in figures, H_2O_2 was measured in the presence of 250 units ml^{-1} of catalase.

2.5.5 Visualisation of superoxide and hydrogen peroxide in tissue

At the tissue level, areas of ROS production were identified by incubating sections of embryonic axes in either 5 mM NBT for 5 min or 3,3'-diaminobenzidine (DAB) for 10 min (Thordal-Christensen *et al.*, 2007). Dark purple staining in the presence of NBT indicates $O_2^{\bullet -}$ production (Serrano *et al.*, 1994) and brown staining in the presence of DAB indicates polymerization of DAB, requiring H_2O_2 and ECPOX activity (Fielding and Hall, 1978).

Excised *C. sativa* embryonic axes were either immediately placed in NBT solution to indicate the location of wounding-induced $O_2^{\bullet -}$ production or, to indicate the location of $O_2^{\bullet -}$ production induced by desiccation, were placed in a humidity chamber, as above in section 2.3, for 16 h to allow for wounding-induced $O_2^{\bullet -}$ production to subside. Embryonic axes were then desiccated in the air of a flow bench (*c.* 0.45 m s^{-1}) for up to 8 h before incubating in NBT. Isolated *P. sativum* seedling axes were desiccated over silica gel for 2 h before incubation in NBT.

2.6 Cell wall fractionation

Cell walls were isolated and proteins fractionated by a modified method of Rast *et al.*, (2003). For the fractionation of *C. sativa* embryonic axes and cotyledons 0.2 g and 1.0 g DW, respectively, of lyophilised material were used for each replicate. Fractionations of *P. sativum* seedling axes were either made from fresh (non-lyophilised) or lyophilised material, as indicated in the figure legends. Between 0.20 – 0.34 g of lyophilised material or 1.2 – 1.4 g of fresh seedlings were used for each replicate. Material was vortexed for ~10 s with 10 ml of ice-cold 0.25 M Tris-HCl buffer at pH 8.0, and centrifuged. All

centrifugation steps were carried out at 4,000 *g* for 15 min at 4 °C. The first supernatant contained the crude cell extract ("C") with the pellet forming the crude cell wall (CW) fraction.

At this stage, depending upon the desired enzymes, three variations were followed in the method. For the collection of B1, B2, B3 and B4 fractions separately, the cell wall pellet (CWP) was re-suspended in 12 ml of 50 mM sodium phosphate buffer at pH 7.0 (P-buffer) and centrifuged. The resulting "B1" supernatant contained loosely associated cell wall proteins. This step was repeated three times, and the supernatants combined. Proteins bound to the cell wall by hydrophobic interactions were then isolated by gently stirring the CWP from the previous step with 10 ml of P-buffer containing 0.3 % (w/v) digitonin for 3 h over ice followed by centrifugation ("B2"; repeated twice). The CWP was re-suspended in 10 ml of P-buffer containing 2 M NaCl, gently stirred for 3 h over ice and centrifuged, releasing proteins bound by ionic bonds into the supernatant ("B3"; repeated twice). Proteins bound by covalent linkages that remained on cell wall fragments ("B4") were re-suspended in 5 ml of P-buffer. The fractionation of *P. sativum* seed coats, cotyledons and embryonic axes separately, as shown in one table only, is courtesy of Miss Mariyana Ivanova (School of Botany, Universtiy Kwa-Zulu Natal, South Africa).

When all removable extracellular enzymes were extracted together (cell wall fractions B1 – B3) the C fraction was removed first and the CWP was incubated in the presence of digitonin and NaCl in 10 ml P-buffer over ice for 3 h, centrifuged and the step was repeated twice.

When only B3 enzymes for protein sequencing were extracted, the C and B1 fractions were removed first. The cell wall pellet was suspended in 2 M NaCl for 5 h over ice and centrifuged as above. The supernatant was de-salted by dialysis using tubing (BioDesign, USA) with 3.5 kD MW pore size against 2 litres of P-buffer at 4 ^{o}C, for 4 h and then overnight in fresh buffer. The desalted sample was concentrated to *c.* 200 µl in volume using Amicon

Ultra-4 centrifugal filter devices (Millipore, USA) with a 3 kD MW pore size. Ten µl of sample, mixed with 1:1 loading buffer, was placed in each well (section 2.7).

Before enzyme measurements, all fractions were centrifuged for 10 min at 12,000 *g* before addition of reagents, except fraction B4, which was centrifuged just before measurement. The B4 fraction contains un-removable cell wall enzymes and measurements were taken of the suspended cell wall pellet.

2.7 Enzyme activities

2.7.1 Peroxidases

Cell fractions of *C. sativa*, *P. sativum* and the leachates of *P. sativum* seedlings were tested for POX activity. The activity was determined spectrophotometrically at A_{420} by following the H_2O_2-dependent oxidation of 2,2'-azino-bis 3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS) to the stable cation radical ($ABTS^{\bullet+}$), using an extinction coefficient of the product of 36 mM^{-1} cm^{-1} (Keesey, 1987).

To assess the activity of POX in cellular fractions, 5 – 100 µl of fraction depending upon POX concentration, were made up to 800 µl with 50 mM P-buffer at pH 6.0, mixed with 100 µl of 10 mM ABTS and 100 µl of 100 mM H_2O_2 (reaction volume = 1 ml) and incubated for up to 10 min at 20 ^{o}C. The activity of ECPOX in the incubation solution (5 ml) containing various sized *P. sativum* seedling axes or wounded seedling axes was measured as before.

The ECPOX activity of the B3 fraction used for sequencing and for $O_2^{\bullet-}$ production was measured by extrapolation against a standard curve created from various concentrations of horseradish POX (HRPOX), which showed a linear relationship between units and concentration up to, at least, 50 units ml^{-1} in the presence of 10 mM H_2O_2. The amount of ECPOX in the B3 fraction was 2.5 ± 0.31 units ml^{-1} and 25.4 ± 4.2 units ml^{-1} before and after concentration, respectively.

Two stains were used for identifying ECPOX in-gel after PAGE; guaiacol (R.P. Beckett, personal communication) and 3,3',5,5'-tetramethyl-benzidine (TMB) (Thomas *et al.*, 1976). Proteins were first re-natured for 1 h in 0.25 M sodium acetate buffer, pH 5.0, containing 10 % glycerol. Gels were incubated in the same buffer, either containing 20 mM guaiacol and 20 mM H_2O_2 that produced orange bands in the presence of ECPOX, or 6.3 mM TMB and 30 mM H_2O_2 that produced blue bands in the presence of ECPOX.

2.7.2 Amine oxidases

To assess the activity of AOX a H_2O_2-dependent reaction coupled to HRPOX in the presence of ABTS was developed and measured spectrophotometrically at A_{420} as in 2.7.1. The reaction was dependent upon H_2O_2 production by AOX in the presence of putrescine. For measuring the activity of cellular fractions, the assay contained 25 µl of fraction, 1100 µl 50 mM K- buffer, 150 µl of 10 mM ABTS, 150 µl of 10 mM putrescine and 75 µl of 750 µg ml^{-1} HRPOX (reaction volume = 1.5 ml) and was incubated for up to 10 min at 20 ^{O}C.

Two stains were used for identifying AOX in-gel after PAGE (see section 2.8), both coupled to the H_2O_2-dependent activity of HRPOX. Proteins were first re-natured for 1 h in 0.1 M P-buffer at pH 7.0, containing 10 % glycerol. Gels were then incubated in 20 ml of the same buffer containing 15 mg of putrescine, 1.5 mg HRPOX and 1 mM diansidine or 20 mM guaiacol, as indicated in the figure legend.

2.7.3 Lipoxygenases

Lipoxygenases were identified in-gel after PAGE by incubation in 0.1 M TRIS-HCl at pH 7.0, 2 mM linoleic acid dissolved in a drop of Triton-X 100 and 1 mM diansidine (De Lumen and Kazeniac, 1976). To test the effect of linoleic or linolenic acid of $O_2^{\bullet -}$ production by wounded *P. sativum* seedling axes, stock solutions (10 mM) were made by dissolving the fatty acids in 1 ml H_2O containing a drop of Triton-X 100 and vortexing for 10 s.

2.7.4 Glucose-6-phosphate dehydrogenase

The activity of glucose-6-phosphate dehydrogenase (G6PDH) was used as a marker of cytosolic contamination of *C. sativa* cell wall fractions. The assay (Kranner, 1998) contained 0.4 ml of fraction, 1 ml of 0.15 M tricine buffer at pH 8.0, containing 0.15 M $MgCl_2$ and 0.15 % G6P. The reaction was initiated by the addition of 0.1 ml of 1.5 % NADP. The change in absorbance at A_{340} was followed for 11 min. Contamination with cytoplasmic enzymes was estimated using the percentage of G6PDH activity in the B1 fraction relative to the activity in the C fraction (n = 4). The activity of G6PDH was also measured in the leachate of wounded *P. sativum* seedling axes for comparison of cytosolic components to the activities of released AOX and ECPOX.

2.7.5 Enzyme Inhibitors

Three enzyme inhibitors were used to assess the contribution of enzymes to the production of ROS. Up to 10 µM diphenylene iodonium (DPI) was used to inhibit flavin-containing NA(P)H oxidases (Henderson & Chappell, 1996), up to 1 mM sodium azide (NaN_3) was used to inhibit hemoproteins, such as ECPOX (Liu *et al.*, 2006), and AOX were inhibited in the presence of up to 5 mM 2-bromoethylamine (2-Br) (Medda *et al.*, 1997; Rea *et al.*, 2002).

Castanea sativa embryonic axes were excised from non-desiccated seeds and immediately placed in either DPI or NaN_3 for 10 min, followed by incubation in epinephrine for 30 min and rates of $O_2^{\bullet-}$ production were measured as in section 2.5.1. To assess the involvement of $O_2^{\bullet-}$-producing enzymes released into the extracellular medium by wounded *C. sativa* embryonic axes, leachates were derived by incubating embryonic axes in H_2O for 1 h, the embryonic axes were removed and $O_2^{\bullet-}$ production was measured after 30 min incubation. The effect of DPI on $O_2^{\bullet-}$ production by the leachates was not tested, because the $gp91^{phox}$ subunit of the NADPH oxidase is an integral membrane-bound protein (Keller *et al.*, 1998), unlikely to be leaked into the incubation medium. The production of $O_2^{\bullet-}$ by

desiccated *P. sativum* seeds during the first 30 min of imbibition and in seedlings 48 h after imbibition was initiated, was measured in the presence of DPI and NaN_3. Measurements of $O_2^{\bullet -}$ and H_2O_2 production, as described in section 2.5.1 and 2.5.4, respectively, by wounded *P. sativum* seedling axes were made during 10 min incubation in the presence of DPI, NaN_3 and 2-Br.

2.7.6 The production of superoxide by ionically bound cell wall enzymes in the presence of hydrogen peroxide

The reaction volume (1 ml) contained 80 µl of B3 fraction, containing 0.2 ECPOX units ml^{-1}, 100 µl of 10 mM epinephrine, 0, 25, 50, 100 or 250 µl of 1 mM H_2O_2 diluted in 50 mM TRIS-HCl buffer at pH 7.0 and made up with 570 – 820 µl of the same buffer. Identical incubations of the same H_2O_2 concentrations were made that also included 0.5 mM $MnCl_2$ dissolved in the buffer. The reaction was initiated with the simultaneous addition of epinephrine and B3 fraction to the incubation solution and $O_2^{\bullet -}$ production measurements were taken after 12 min, as in section 2.5.1. Measurements of $O_2^{\bullet -}$ were also made 5, 10, 15 and 20 min during the incubation of the B3 fraction with 50 µl of H_2O_2. Separate incubations were set up to measure H_2O_2 consumption at the same time intervals.

2.8 Electrophoretic separation of proteins

Cell wall fractions were concentrated over night at 4 ^oC by dialysis against 20 % polyethylene glycol 6,000, using tubing with 3.5 kD MW pore size (BioDesign, USA) before separation by PAGE. Electrophoretic studies were carried out using a modification of the method of Laemmli (1970). Stacking and separating gels were 4 and 10 % polyacrylamide, respectively. Running buffer and gels contained 0.1% sodium dodecyl sulphate (SDS), but samples were not heated, and mercaptoethanol and SDS were omitted from the loading buffer. Samples were run at 120 V until the dye-front reached the separating gel and then for up to 3 h at 150 V. Molecular mass markers (Sigma, USA) were stained with Coomassie brilliant blue, G-250.

2.9 Protein sequencing of superoxide-producing enzymes

Proteins were sequenced, courtesy of Dr Lucas Bowler, TCMR, University Sussex, UK, according to a modified method of Shevchenko et al., (1996). Briefly, the NBT-stained bands were rinsed with acetonitrile and then reduced by 10 mM DTT in 25 mM NH_4HCO_3 by heating at 56 ^oC for 60 min. Following centrifugation the DTT solution was removed and 50 µl of 55 mM iodoacetamide and 25 mM NH_4HCO_3 was added to the sample and incubated for 45 min in the dark. After centrifugation the iodoacetamide was removed and the bands were rinsed again in acetonitrile and 25 mM NH_4HCO_3 (x 2). The gel pieces were then completely dried in a vacuum desiccator. Modified porcine trypsin (Promega) was added to the sample in 50 µl of 5 mM NH_4HCO_3, using a protein-to-trypsin ratio of 100:1 (w/w), placed on ice for 45 min then incubated overnight at 37 ^oC. The reaction was terminated by the addition of 1 % (v/v) trifluoroacetic acid. Proteins were extracted by sonicating the gel for 5 min in 50 µl 20 mM NH_4HCO_3. After centrifugation the supernatant was removed and the step was repeated twice, but using 50 µl of 5 % formic acid and 50 % acetonitrile. The pooled supernatant were concentrated to 25 µl using a vacuum desiccator and stored at -20 ^oC until used. For MS, samples were diluted 1:10 with 0.1% trifluoroacetic acid and 5 – 10 µl were loaded.

Resulting digests were then fractionated on a 150 mm x 0.075 mm reverse phase column (PepMap100 C18, Dionex, CA) using an Ultimate U3000 nano-LC system (Dionex, CA) equipped with a 20 µl injection loop. Samples were run on a C18 reverse phase column (PepMap 100, Dionex) using an Ultimate U3000 nano-LC system (Dionex) equipped with a 20 µl injection loop. Peptide separation was performed using a linear gradient from 100 % solvent A (water, acetonitrile, formic acid; 97.9:2.0:0.1 v:v) to 60 % solvent B (acetonitrile, water, formic acid; 90:9.9:0.1 v:v) at a flow rate of 350 nl min^{-1}. Eluting peptides were directly analysed by tandem mass spectrometry using a LTQ Orbitrap FT-MS (ThermoScientific) fitted with a nanospray ion source and using stainless steel nano-bore emitters (both Proxeon Biosystems, Odense).

Tandem mass spectra were collected in a data-dependent fashion by collecting one full MS scan in the Orbitrap detector (FT) (m/z range: 350-1800) followed by MS/MS spectra of the six most abundant precursor ions (in ion trap). Lock-mass to ensure mass accuracy (by real-time recalibration of masses) was enabled on the Orbitrap, using a background ion (polymethylcyclosiloxane, m/z 429.0887) naturally generated during the electrospray process.

The resulting MS/MS spectra were then used to search against an annotated database (Swissprot) using the SEQUEST protein identification algorithm as implemented within BioWorks v3.3 (Thermo Scientific). Stringent filtering criteria used for positive protein identifications were Xcorr values greater than 1.9 for +1 spectra, 2.2 for +2 spectra and 3.75 for +3 spectra and a delta correlation cutoff of 0.1.

2.10 Identification of Low-molecular-weight compounds in wounded *Pisum sativum* leachates

Low-molecular-weight compounds in the leachates of wounded seedling axes were identified, courtesy of Dr. Louise Colville, SCD, RBG Kew, by gas chromatography-mass spectroscopy (GC-MS). Seedlings were rinsed in dH_2O before seedling axes were excised. Twenty axes were incubated in 10 ml dH_2O at 20 ^{o}C for 5 min with constant agitation (75 rpm). The leachate was transferred to a new vial, frozen in liquid nitrogen and stored at -75 ^{o}C prior to lyophilisation. The freeze-dried residue was derivatised with 0.2 ml N,O-bis-(trimethylsilyl)-trifluoroacetamide and 0.3 ml pyridine at 70 ^{O}C for 30 min then centrifuged at 12,000 *g* for 5 min. The supernatant was analysed using GC (Thermo Finnigan Trace GC Ultra) with a Rtx-5MS column (15 m length, 0.25 mm internal diameter, 0.25 mm df; Restek) running a temperature program (1 min at 80 ^{o}C, 15 ^{o}C min^{-1} to 280 ^{o}C, 6 min hold; helium carrier gas at constant flow rate of 1 ml min^{-1}). A 1 µl injection volume was used and compounds were detected using MS (Thermo Finnigan Trace DSQ; ionization

energy 70 eV, scan frequency range *m/z* 10-1000 per 0.2 s), and identified through comparison with the NIST mass spectral database.

2.11 Identification of polyamines in wounded *Pisum sativum* leachates

Polyamines were detected in leachates of wounded *P. sativum* seedlings using the method of Flores and Galston (1982). Seedlings were rinsed in dH_2O, excised as before and 10 axes were incubated in 2 ml^{-1} dH_2O at 20 ^oC for 10 min. The leachate was freeze dried overnight and embryonic axes were taken for DW measurements. The freeze-dried extract was vortexed with 125 µl of ice cold 5 % perchloric acid ($HClO_4$) for 10 s. To derivatise the polyamines, the $HClO_4$ extract was vortexed for 20 s with 0.5 ml of 2 M NaOH and 10 µl of benzoyl chloride before incubating at room temperature. After 20 min, 0.5 ml of saturated NaCl solution was added and benzoyl-polyamines were extracted in 1 ml of diethyl ether after vigorous vortexing and centrifugation at 1500 *g* for 1 min. 800 µl of the ether phase was recovered and incompletely dried in a stream of nitrogen for several min before being suspended in 200 µl methanol. If the sample was completely dried it became irremovable from the vial and polyamines were not detected. 10 µl of methanol-suspended sample was separated isocratically using HPLC and an RP-18 column. The solvent consisted of 64 % methanol in dH_2O. Benzoyl-polyamines were detected at an absorbance of 245 nm during 20 min run time and measurements were calculated against standards containing up to 50 nmol of polyamine in the $HClO_4$ extract. The peaks of spermine, putrescine and agmatine standards were wide and overlying and unfortunately individual polyamines could not be identified, but consistent standard peak sizes allowed the total polyamines in the leachate to be calculated.

2.12 Statistical analysis

Data were tested for significance using one or two way analysis of variance (ANOVA) in combination with post-hoc comparison of means using the Least

Significant Difference (LSD) test. Arcsine transformation was applied to percentage data before analysis. ANOVA relies on the assumption that the data being analysed is normally distributed. When using percentage values that are, by their very nature, restricted to the interval of 0 to 100, arcsine transformation is a method of correcting percentage values close to interval limits to simulate a normal distribution of data prior to ANOVA. In figures, columns and data points or table values labelled with the same letters do not differ significantly ($P < 0.05$). When upper and lower cases are shown together in the same figure ANOVA was run separately for each letter case. All error bars denote standard error.

Chapter 3 Effect of embryonic axis excision and desiccation on superoxide production by *Castanea sativa* embryonic axes

3.1 Introduction

Recalcitrant seeds are intolerant to desiccation, hence can not be stored at low WCs and, therefore, at temperatures that induce freezing. Storage at high WC facilitates the growth of micro-organisms leading to critical infections (Berjak and Pammenter, 2008). Therefore, improving long term storage methods of recalcitrant seeds is pertinent for conservation especially considering in some diminishing habitats, such as tropical forests, up to 48 % of seed-bearing plants produce recalcitrant seeds (Tweddle *et al*., 2003). So far, cryopreservation protocols have been developed for just a handful of species and there are still major challenges regarding efficiency and applicability of cryopreservation protocols to a wider range of taxa (Engelmann, 2004). Although cryopreservation protocols have been established for *C. sativa* embryonic axes (Pence, 1992, Corredoira *et al*., 2004), the protocol curtails many stresses. On a biochemical level, the stress responses during individual stages of cryopreservation are poorly understood. Characterising the stresses could lead to improving the storage and recovery of difficult-to-handle seeds.

The first stage to cryopreserving large fleshy seeds requires excising the embryonic axis from the seed. Isolated embryonic axes can be cultivated by replacing seed reserves with a suitable growth medium (Norstog, 1973). It has been noted that the proximity of the cut surface to the excised axis influences shoot development (Goveia *et al*., 2004). Wounding releases polyphenol oxidases (laccases and catechol oxidases), which oxidise flavanoids (bi-phenolic molecules) to heterogeneous polymers, thereby creating cytotoxic compounds, rendering the tissue unpalatable (*reviewed in* Pourcel *et al*., 2006). Recalcitrant palm species showed retarded growth when

oxidation-related browning followed axis excision (Davies, 2008). Following excision, it is critical for the embryonic axis to be desiccated sufficiently to prevent nucleic ice crystallisation from rupturing cells during cryopreservation. A trade off exists between ice crystallisation at high WC and desiccation stress at low WC with a small hydration window in between when axis viability can be maintained if rapidly cooled in liquid nitrogen (Dussert *et al.*, 2002; Corredoira *et al.*, 2004).

Desiccation to a sufficiently low WC is metabolically stressful, which can be minimised by rapid desiccation rates (Berjak *et al.*, 1984; Pritchard, 1991; Vertucci and Farrant, 1995). Due to the small size of embryonic axes (often just a few mm^3), desiccation, freezing and thawing rates are faster compared to whole seeds. Recalcitrant seeds exhibit limited control of metabolism at reduced WC (Leprince *et al.*, 1999; Franks and Drake, 2003), and ongoing metabolism in a water-reduced environment leads to enhanced electron leakage from mitochondrial electron transport chains (Inze and Van Montagu, 1995; del Rio *et al.*, 2002), enhancing the production of ROS (Hendry, 1993; Finch-Savage *et al.*, 1994; Côme and Corbineau, 1996). Oxidative attack during desiccation can cause damage to essential molecules such as proteins, lipids and nucleic acids (Smirnoff, 1993; Evans *et al.*, 2004; McCord, 2008). An indication of the level of oxidative stress can be gained by measuring the redox state of major cellular antioxidants (Kranner and Birtić, 2005). Furthermore, using the concentrations of oxidised and reduced forms of a redox couple, such as GSH and GSSG, the $E_{GSSG/2GSH}$ can be calculated, providing an reliable viability marker (Schafer and Buettner, 2001; Kranner *et al.*, 2006). Combining $E_{GSSG/2GSH}$ with other viability assessments following various desiccation intervals could provide a powerful tool for understanding desiccation related seed death.

Despite the detrimental effects of oxidative stress, ROS can also serve many beneficial roles. Abiotic stresses often stimulate an enzymatic production of extracellular ROS (de Bruxelles and Roberts, 2001; Beckett *et al.*, 2004; Minibyeva *et al.*, 2009), which feed into signalling networks

initiating responses (Kovtun *et al*., 2000; Leon *et al*., 2001; Laloi *et al*., 2004), such as transcription (Desikan *et al*., 2001; Orozco-Cardenas *et al*., 2001) and programmed cell death (Raff, 1998; Hengartner, 2000; Gechev *et al*., 2006). Wounded tissue provides an entrance to pathogens and the localised production of ROS at the wound site may also be involved in rapidly rebuilding damaged cell walls (Bradley *et al*., 1992; Bernards *et al*., 2004). Therefore, wounding and desiccating embryonic axes may both stimulate the beneficial production of extracellular ROS in addition to the uncontrolled and damaging desiccation-induced intracellular formation of ROS.

Hypothesising that oxidative stress is a major cause of desiccation-induced seed viability loss, the concentrations and $E_{RSSR/2RSH}$ of LMW thiols and their corresponding disulphides were measured and correlated to germination and viability. The leakage of solutes, indicative of membrane damage, by embryonic axes was measured at point of viability loss to indicate whether oxidative stress has caused damage. The wounding-induced production of $O_2^{\bullet-}$ by the embryonic axis was quantified for up to 8 h following embryonic axis isolation. Extracellular $O_2^{\bullet-}$ production by axes was measured following both rapid desiccation and slower in-seed desiccation. The former is generally utilised during cryopreservation protocols and the later may be encountered in more natural field like conditions.

3.2 Results

Two accessions of seed were used for all experiments and the desiccation effect on viability for both is provided. The WC of the commercially available seed lot in 2006 and 2007 was in the region of 10 % and 15 % lower than previously published for the WC of *C. sativa* at shedding (Corredoira *et al*., 2004), suggesting that the seeds had slightly dried out during transport. Therefore, seeds were soaked before storage to reduce the chance of desiccation damage before further experimentation.

3.2.1 Seed water content, germination and embryonic axis viability

Seeds 2006

The initial WC of the axis and cotyledon was 53 ± 2 and 46 ± 1 % respectively (Fig. 3.1A). Soaking untreated seeds with pericarp attached for 48 hours increased the WC in the embryonic axes and cotyledons to 61 ± 2 % and 50 ± 2 %, respectively. Soaked seeds displayed increased vigour, assessed by germinating 3 d after sowing compared to 6 d in un-soaked seeds (Fig. 3.1B). Soaking seeds without pericarp for just 1 h gave the same increase in WC and germination response as soaking seed with complete pericarp for 48 h, but for practicalities and preventing transmission of pathogens, all seed was soaked with pericarp attached before storage and further experiments. As the pericarp was shown to be a barrier to water uptake, it would also inhibit water loss and was removed before all seed desiccation to enhance uniform WC at each desiccation interval. When desiccated at 15 % RH and 15 ^{O}C within intact seeds, embryonic axes reached a WC of 20 % after 10 d (Fig. 3.1A). Isolated axes dried much faster, reaching 20 % WC after about 5 h (Fig. 3.1A).

A rapid decrease in total germination was observed following desiccation of intact seeds (Fig. 3.1B). Seeds desiccated for just 3 d to an axis WC of 30.1 ± 2.6 % lost the ability to germinate. However, isolated embryonic axes desiccated to a similar WC of 32 % still retained almost 50 %

viability (Fig. 3.1A; 3.1C). Figure 3.1C suggests that after 21 d in tissue culture, axes desiccated to 32 % WC had lost vigour, i.e. the surviving axes showed only about 70 % of the axis elongation found in controls (Fig. 3.1C). Overall, the vigour of isolated axes, assessed by axis elongation, was negatively correlated (R^2 = 0.92, P = 0.02) with WCs of 38 % and below (Fig. 3.1C).

Seeds 2007

The initial WC of axes and cotyledons were 48.9 ± 2.2 and 45.3 ± 1.6 % respectively (Fig. 3.2A). Total germination of untreated seeds was 60 ± 3 %, increasing to 75 ± 9 % by soaking seeds (with pericarp removed) in H_2O for 1 h before germination (Fig. 3.2B), thereby increasing seed WC by 3 %. After 5 d of desiccation, the WC fell to 28 ± 3 % and 31 ± 4 % in the cotyledons and the embryonic axes, respectively. At this point, the intact seeds had lost their ability to germinate. However, 90 % of the axes could be rescued by aseptic tissue culture (Fig. 3.2B). After longer times of desiccation, the axes also progressively lost viability. Desiccation for 21 d decreased embryonic axis WC to 24 ± 2 % and the viability fell by three quarters compared with non-desiccated controls.

Comparison of germination and viability of seeds from 2006 and 2007

Castanea sativa seeds are prone to microbial contamination, although germination tests revealed heavy infections were not always critical (Fig. 3.3A). The effect of desiccation on germination of seeds from 2006 and 2007 was similar, although the viability of isolated embryonic axes varied (Fig. 3.3B).

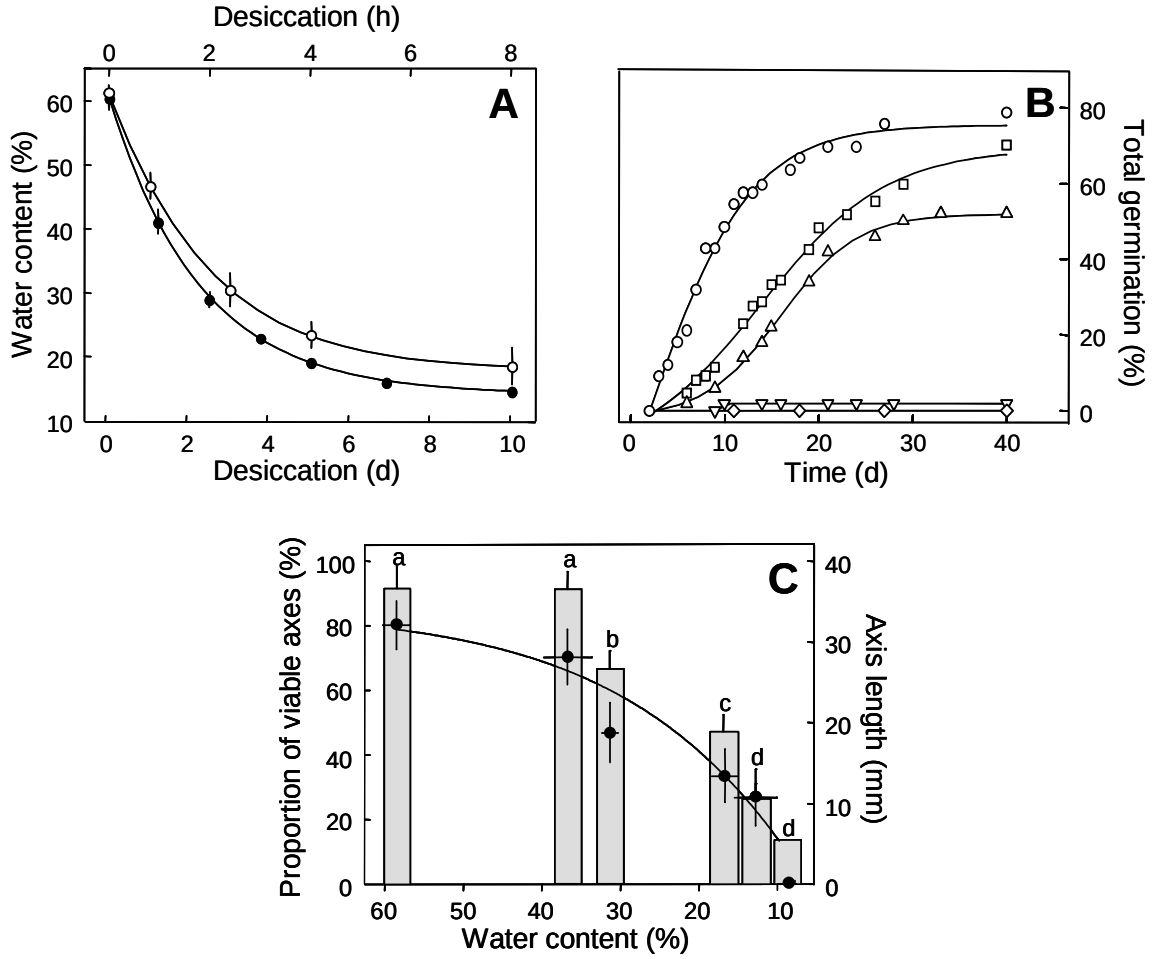


Figure 3.1 Effect of desiccation on *Castanea sativa* seeds and embryonic axes, 2006. **[A]** Decline in water content in embryonic axes that were isolated following desiccation of intact *Castanea sativa* seeds (bottom x-axis; open symbols; n = 20 ± SE) and in isolated embryonic axes that were excised from intact seeds and desiccated immediately after excision (top x-axis; closed symbols, n = 8 ± SE). **[B]** Total germination of intact seeds immediately after purchase (axis WC = 53 ± 2 %, squares), after soaking in water (WC = 61 ± 1 %, circles) and after desiccation to a range of water contents (upwards triangles, downwards triangle and diamonds represent WC of 46 ± 2 %, 30 ± 3% and 23 ± 2 %, respectively), n = 100 seeds. **[C]** Correlation between axis WC, viability and vigour of isolated embryonic axes courtesy of Mr Ian Green. Axis viability is defined by the proportion of axes displaying growth (left y-axis; circles) and axis vigour is defined by axis length (right y-axis; bars) following 21 d of tissue culture (n = 5 replicates of 5 axes ± SE).

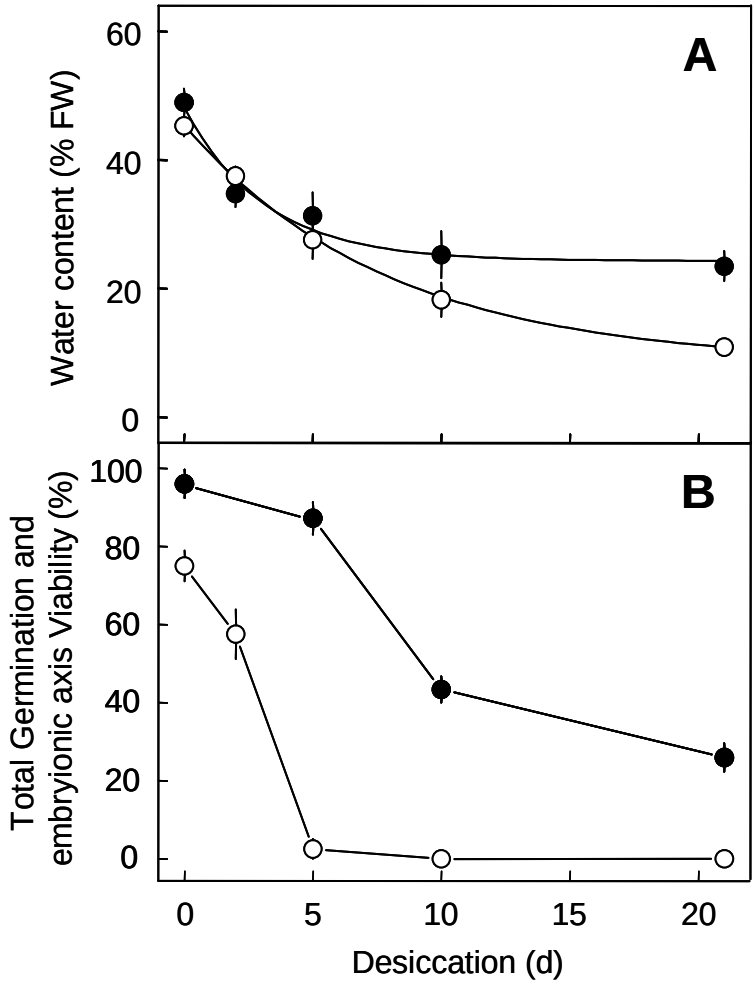


Figure 3.2 Loss of total germination and embryonic axis viability in response to desiccating *Castanea sativa* seeds, 2007. Seeds were desiccated at 15 % RH and 15 ^{O}C. **[A]** Water content in isolated embryonic axes (closed symbols) and cotyledons (open symbols) (n = 4 replicates of 5 axes or cotyledons). **[B]** A direct comparison can be made of total germination (open symbols) and the viability of isolated embryonic axes from seeds that had been desiccated identically (closed symbols). Axis viability is shown as a percentage of axes that expanded to more than 10 mm in length after 21 d in aseptic tissue culture (means ± SE, n = 5 replicates of 8 seeds for total germination and n = 3 replicates of 15 embryonic axes for embryonic axis viability).

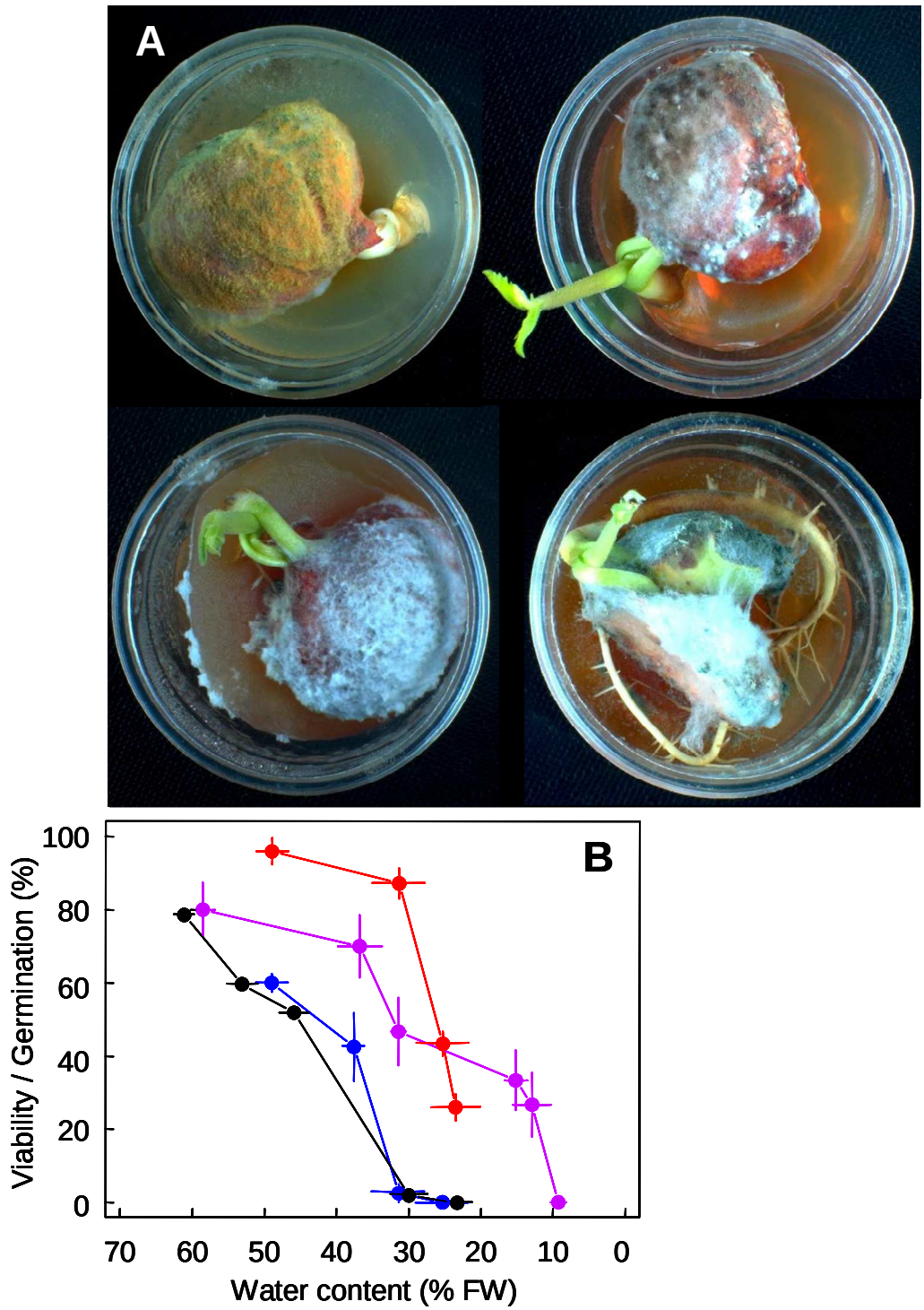


Figure 3.3 Photos of infected *Castanea sativa* seeds during germination and a comparison of 2006 and 2007 seed accession viabilities. **[A]** Ability of *Castanea sativa* seeds to tolerate pathogen challenges during germination. Infected but viable seeds, 2006, were photographed after 21 d germination. **[B]** Comparison of total seed germination and embryonic axis viability from Fig. 3.1 and Fig. 3.2 of seeds collected in 2006 and 2007, respectively. Seeds from 2006 (black) and 2007 (blue) were desiccated at 15 % RH to various WCs before germination. Axes were either isolated from seeds, 2006, before desiccation (purple) or isolated from desiccated seeds, 2007 (red).

3.2.2 The effects of desiccation on non-growth viability markers

Membrane permeability

Electrolyte leakage in axes following drying to 26 % WC was 15-fold higher ($P < 0.001$) after 6 h of measurement as compared with control (Fig. 3.4), suggesting that membrane permeability had changed significantly. This shows that membrane leakiness occurred at a WC associated with the onset of viability loss, which became statistically significant at WCs of 32 % and below (Fig. 3.1C).

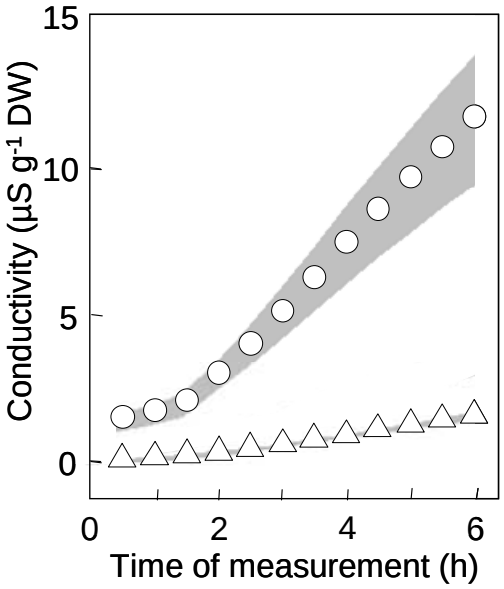


Figure 3.4 Desiccation induced changes in membrane permeability in isolated embryonic axes of *Castanea sativa* seeds, 2006, as assessed by electrolyte leakage. Axes were excised and either desiccated (circles) or kept hydrated (triangles) for 6 h to a WC of 26 ± 3 % or 66 ± 2 %, respectively. Conductivity measurements were taken every 30 min for 6 h (n = 6 replicates of 3 axes each that were incubated in 1.5 ml of H_2O, grey shading represents SE).

The half-cell reduction potentials of low-molecular-weight thiol – disulphide redox couples

On a DW basis, axes of non-desiccated seeds contained approximately twice as much total LMW thiols than cotyledons (Fig. 3.5). Of the total LMW thiols and corresponding disulphides of non-desiccated axes and cotyledons, 94 % and 87 %, respectively, was total glutathione (Fig. 3.5). In non-desiccated seeds all $E_{RSSR/2RSH}$ except $E_{cys(ox)/2cys(red)}$ had non-significantly ($P < 0.05$) different values in the axis and cotyledon. During desiccation each of the LMW thiols became oxidised and depleted. Correspondingly, the $E_{RSSR/2RSH}$

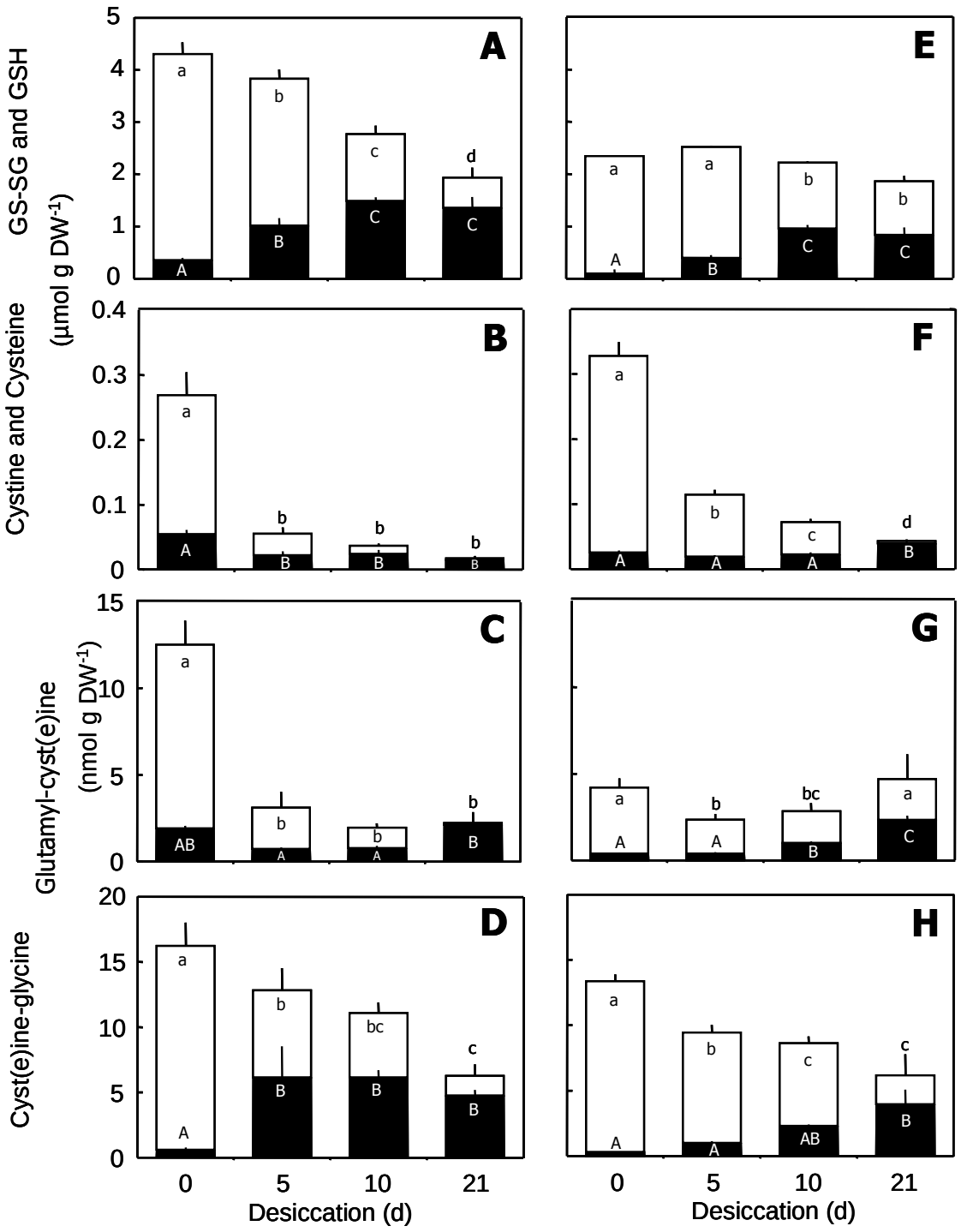


Figure 3.5 Oxidation of LMW thiols and accumulation of disulphides in *Castanea sativa* seeds, 2007, following desiccation for 0, 5, 10 and 21 d at 15 % RH. Figures **[A - D]** and **[E - H]** denote embryonic axes and cotyledons, respectively. In all figures white bars represent LMW thiols and black bars represent their corresponding disulphides. Data are means + SE (n = 4 reps of 5 axes or 5 cotyledons). Different letters denote significant difference calculated separately for LMW thiols and disulphides and at each desiccation interval.

increased towards more positive (more oxidizing) values (Fig. 3.5). After 5 d of desiccation, a rapid loss of cysteine, the second most abundant LMW thiol, occurred in the embryonic axis (Fig. 3.5C), which increased the representation of total glutathione within all LMW thiols and their corresponding disulphides. In embryonic axes, but not cotyledons, concentrations of GSH decreased more than the concentrations of GSSG increased, suggesting a major loss of glutathione during desiccation.

After 5 d desiccation there were limited losses in embryonic axis viability (Fig. 3.2B), but the intracellular redox environment, as indicated by the change in $E_{RSSR/RSH}$, had already become more oxidised (Fig. 3.6). Following 21 d desiccation, the increase in all measured $E_{RSSR/RSH}$ of the axis were between 69 and 76 mV, whereas in the cotyledon the range of increase was much greater, e.g. the increase in $E_{GSSG/2GSH}$ was only 22 mV compared to 101 mV increase in $E_{cys(ox)/2cys(red)}$ (Fig. 3.6). Note that $E_{RSSR/2RSH}$ is a function of the molar concentrations that are determined by WC, which after 21 d desiccation was twice as high in the embryonic axes as in the cotyledons. As a consequence, in conjunction with a higher thiol : disulphide ratio, the pattern of $R_{RSSR/2RSH}$ during desiccation reflected a more reducing environment in the cotyledons than in embryonic axes. Table 3.1 shows that the $E_{GSSG/2GSH}$ correlated strongly (R^2 = 0.96, P = 0.04) with viability in embryonic axes while the correlation between $E_{GSSR/2GSH}$ of the cotyledons and seed germination was weaker (R^2 = 0.82, P = 0.18), suggesting that total germination and the $E_{GSSG/2GSH}$ of the cotyledons are not suitable indicators of axis viability. Moreover, the low correlation (R^2 = 0.67, P =0.33) of axis $E_{GSSG/2GSH}$ and total germination suggests that other factors than embryonic axis viability were affecting seed germination. Glutathione and cyst(e)ine were the most abundant LMW thiol – disulphide redox pairs identified (Fig. 3.5). Although the standard half cell reduction potential for glutathione is 14 mV more negative than cyst(e)ine (Jones *et al.*, 2000), the $E_{GSSG/2GSH}$ of the embryonic axis was maintained at *c.* 50 mV more negative than the $E_{Cys(ox)/2Cys(red)}$ over 21 d desiccation (Fig. 3.7), which is due to the much greater abundance of GSH (Fig 3.6A).

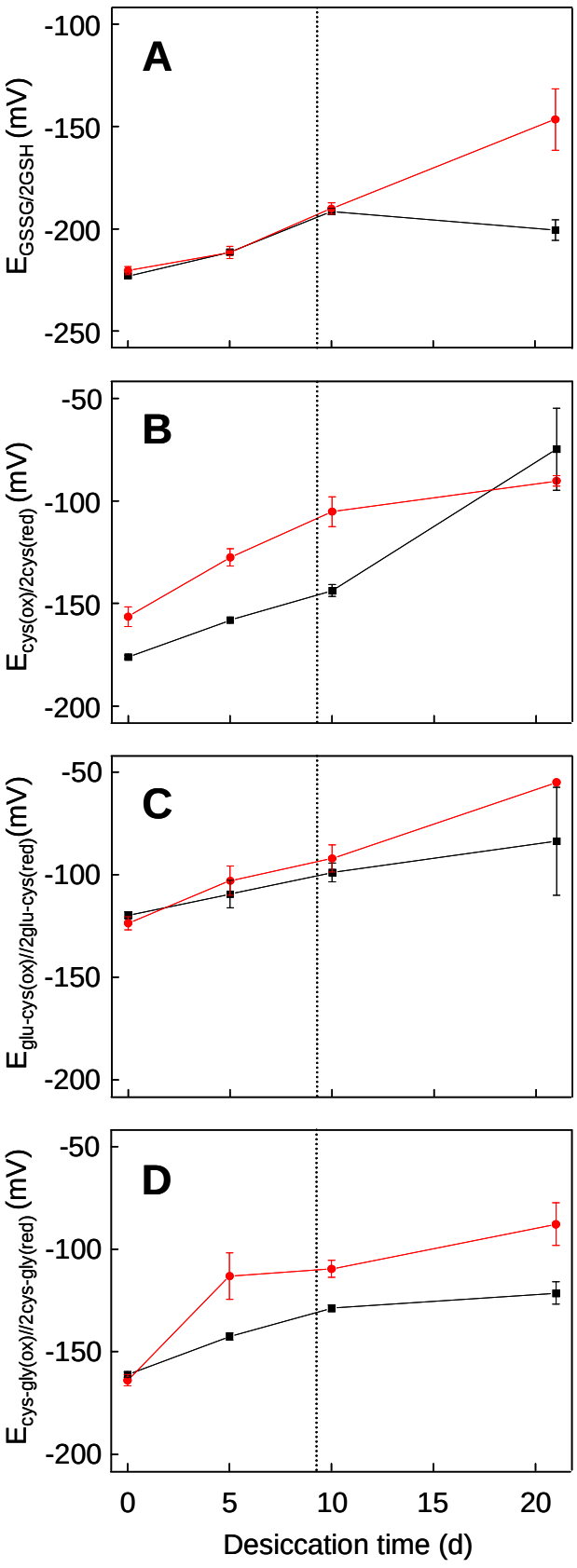


Figure 3.6 Effect of desiccation on the half cell reduction potentials of low-molecular-weight thiols and their corresponding disulphides of *Castanea sativa* seeds, 2007. Seeds were desiccated for up to 21 d. The ($E_{RSSR/2RSH}$) is shown for axes (red lines) and cotyledons (black lines), where R represents **[A]** glutathione; **[B]** cyst(e)ine; **[C]** glutamyl-cyst(e)ine and **[D]** cyst(e)inyl-glycine. The dotted line represents the estimated time of desiccation when embryonic axis viability is at 50 % (see Fig 3.2B). Data are means ± SE (n = 4 replicates of 5 axes or 5 cotyledons).

Table 3.1 Correlation, as expressed as R^2, of $E_{RSSR/2RSH}$ of the two most dominant LMW thiol-disulphide redox pairs with embryonic axis viability or total germination. The probability (P) of a correlation is also shown, with closer values to zero suggesting a greater correlation.

	Axis $E_{RSSR/2RSH}$ Axis viability	Axis $E_{RSSR/2RSH}$ TG	Cot $E_{RSSR/2RSH}$ Axis viability	Cot $E_{RSSR/2RSH}$ TG
Glutathione	R^2 = 0.96 P = 0.04	R^2 = 0.67 P = 0.33	R^2 = 0.85 P = 0.15	R^2 = 0.82 P = 0.18
Cysteine	R^2 = 0.95 P = 0.05	R^2 = 0.87 P = 0.13	R^2 = 0.88 P = 0.12	R^2 = 0.59 P = 0.41

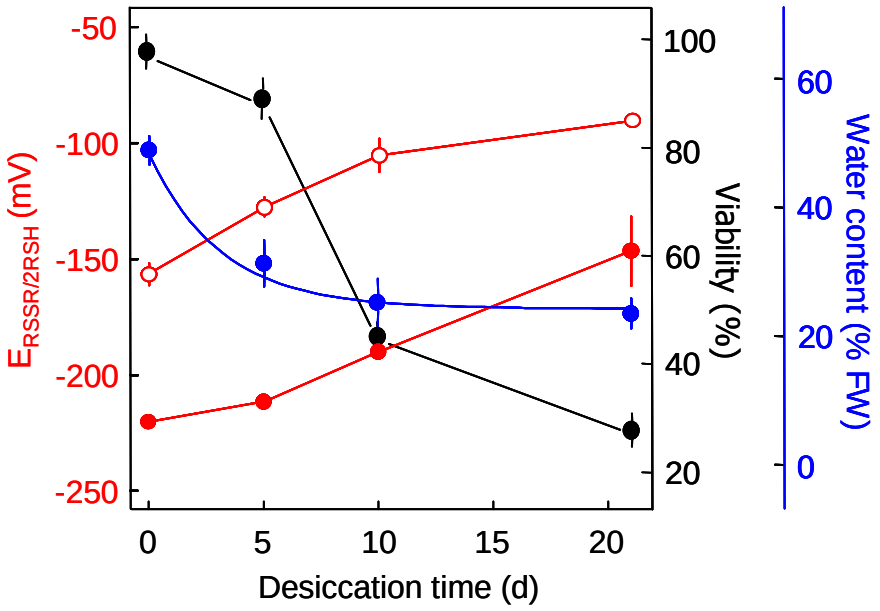


Figure 3.7 Comparison of embryonic axis $E_{RSSR/2RSH}$ for glutathione and cyst(e)ine during the desiccation induced viability loss of *Castanea sativa* seeds at 15 % RH and 15 ^{O}C. The $E_{GSSG/2GSH}$ and $E_{cys(ox)/2cys(red)}$ represented by the filled and open red dots respectively (left y axis) both increase during desiccation with $E_{cys(ox)/2cys(red)}$ staying *c.* 50 mV or above more positive than $E_{GSSG/2GSH}$. The embryonic axis viability is represented by the black dots (right black y axis) taken from Fig. 3.2B. The WC is represented by the blue dots (far right blue y axis) taken from Fig. 3.2A.

3.2.3 Production of superoxide following embryonic axis excision

At the tissue level, $O_2^{\bullet-}$ production was visualised by NBT staining, revealing strict confinement to the cut surface of the embryonic axis (Fig. 3.8A). Quantifying the amount of $O_2^{\bullet-}$ produced using the epinephrine assay showed that in the first 5 min after excision axes of non-desiccated seeds released $O_2^{\bullet-}$ into the incubation medium at a rate of 1.7 nmol g DW^{-1} s^{-1}. When the same embryonic axes were placed into new incubation solutions every 5 min the rates of $O_2^{\bullet-}$ production had dropped to around 0.25 nmol g DW^{-1} s^{-1} between 5 – 10 min and continued at this rate up to 30 min following excision (Fig. 3.8B). When different axes were used for each measurement, after the first 5 min of activity, rates halved to 1 nmol g DW^{-1} s^{-1} by 30 min, declining to almost zero $O_2^{\bullet-}$ production by 8 h (Fig. 3.8C). No significant changes in WC were recorded during moist storage (data not shown). ESR confirmed that axis excision caused a burst of $O_2^{\bullet-}$. The interaction of the unpaired electron of $O_2^{\bullet-}$ with the two non-equivalent ring protons of Tiron produced a typical 4-line Tiron semiquinone spectrum (Fig. 3.8B, inset). The amplitude of the spectra was proportional to the amount of $O_2^{\bullet-}$ produced.

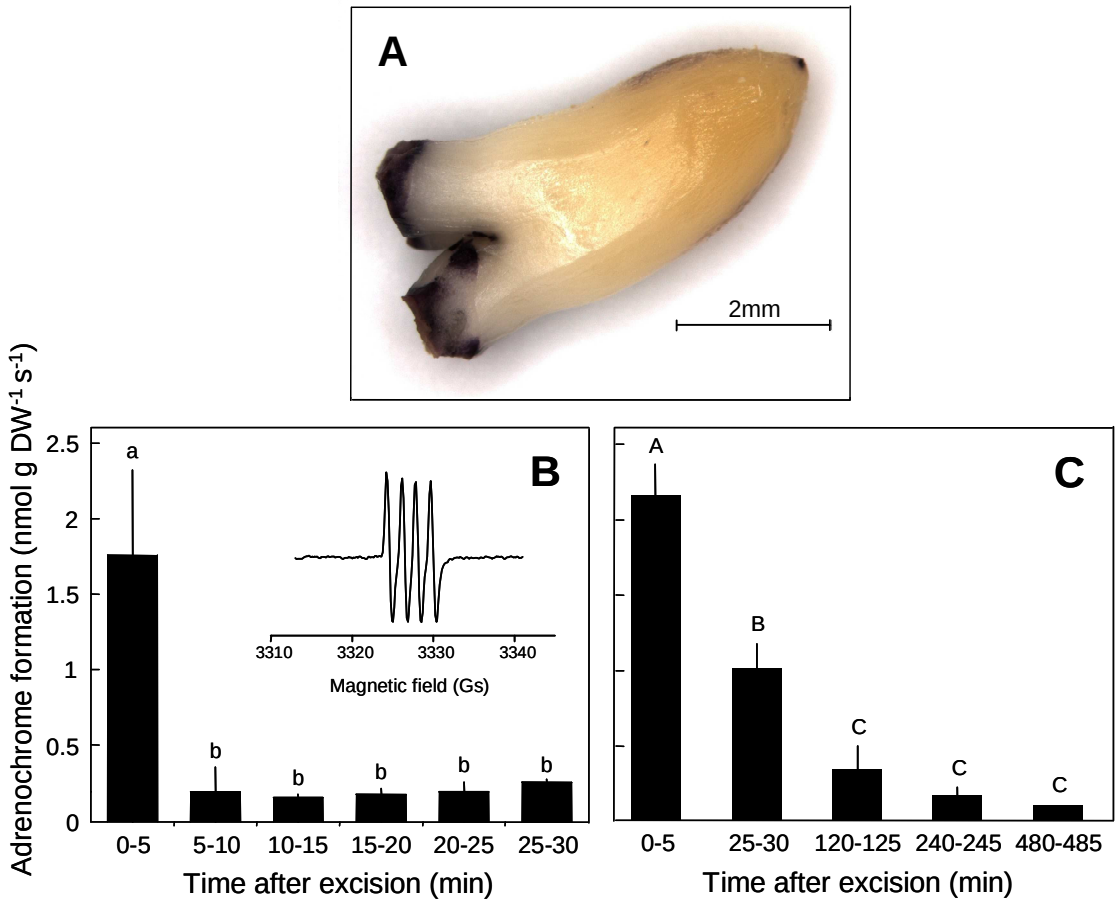


Figure 3.8 Superoxide production following embryonic axis excision from *Castanea sativa* seeds, 2006. **[A]** Superoxide production was visualised immediately after excision in the presence of NBT, which turns dark purple in the presence of superoxide. **[B]** Extracellular $O_2^{\bullet -}$ production was quantified by measuring the oxidation of epinephrine to adrenochrome in 3.5 ml incubation solution. The same plant material was used in all measurements and the incubation solution was changed every 5 min. The $O_2^{\bullet -}$-specific spin trap Tiron was used to confirm $O_2^{\bullet -}$ production by embryonic axes; the inset shows a typical ESR spectrum of the Tiron semiquinone radical formed as a result of $O_2^{\bullet -}$ production, courtesy of Dr Farida Minibayeva. **[C]** Effect of moist storage on $O_2^{\bullet -}$ production by embryonic axes following excision. Different plant material that had been kept in a sealed humidity chamber and fresh incubation solution were used for each measurement. For both [A] and [B] each bar shows the average rate of $O_2^{\bullet -}$ production over 5 min (n = 4 replicates of 5 axes + SE).

3.2.4 The effect of desiccation on superoxide production by isolated embryonic axes

Desiccation of embryonic axis after isolation

In non-desiccated axes, purple NBT staining of $O_2^{\bullet-}$ was confined to the cut surface only (Fig. 3.8A). Following desiccation in an air stream of a flow bench, the stain progressively spread across the surface of the axes (Fig. 3.9A). The intensity of the stain was higher at a WC of 36 % then at higher or lower WC. Desiccating excised axes to 32 % WC reduced viability to *c.* 50 % (Fig. 3.1C) and increased the amount of $O_2^{\bullet-}$ produced within the initial burst (white bars in Fig. 3.9B) compared to mildly desiccated axes (black bars in Fig. 3.8B). Further desiccation to 25 % WC reduced initial rates of $O_2^{\bullet-}$ production (grey bars in Fig. 3.9B). Plotting individual data points for all treatments for the initial burst of $O_2^{\bullet-}$ revealed a hyperbolic relationship between WC and $O_2^{\bullet-}$ production (Fig. 3.9C), which could be deemed typical of a stress response. Fitting two straight lines through the data results in a point of interception at 4 nmol g DW^{-1} s^{-1}, suggesting that excising embryonic axes are capable of maximal $O_2^{\bullet-}$ production in this range.

Desiccation of embryonic axes within seed

A similar trend between WC and $O_2^{\bullet-}$ production was also observed when embryonic axes were desiccated within seeds. The method for determining $O_2^{\bullet-}$ production when embryonic axes were desiccated in-seed were modified from measurements of axes desiccated after isolation by first, rinsing the axes to remove cytoplasmic contamination from the wounded surface and second, imbibing in epinephrine for an interval of 1 h sufficient time to fully rehydrate the axes (data not shown). Rinsed axes isolated from non-desiccated seeds produced $O_2^{\bullet-}$ at a rate of 0.14 ± 0.02 nmol g DW^{-1} s^{-1} (Fig. 3.9D). Production of $O_2^{\bullet-}$ significantly increased in embryonic axes desiccated up to 5 d while viability was maintained (Fig. 3.2B), peaking at a rate of 0.34 ± 0.03 nmol g DW^{-1} s^{-1} (Fig. 3.9D). Both viability and $O_2^{\bullet-}$ production decreased with further desiccation. Overall, regardless of whether embryonic axes were

desiccated rapidly following excision or slower within seeds, a pleiotropic pattern of extracellular $O_2^{\bullet -}$ production to WC was observed, with $O_2^{\bullet -}$ production first increasing with mild desiccation and decreasing with critical desiccation that led to critical viability loss.

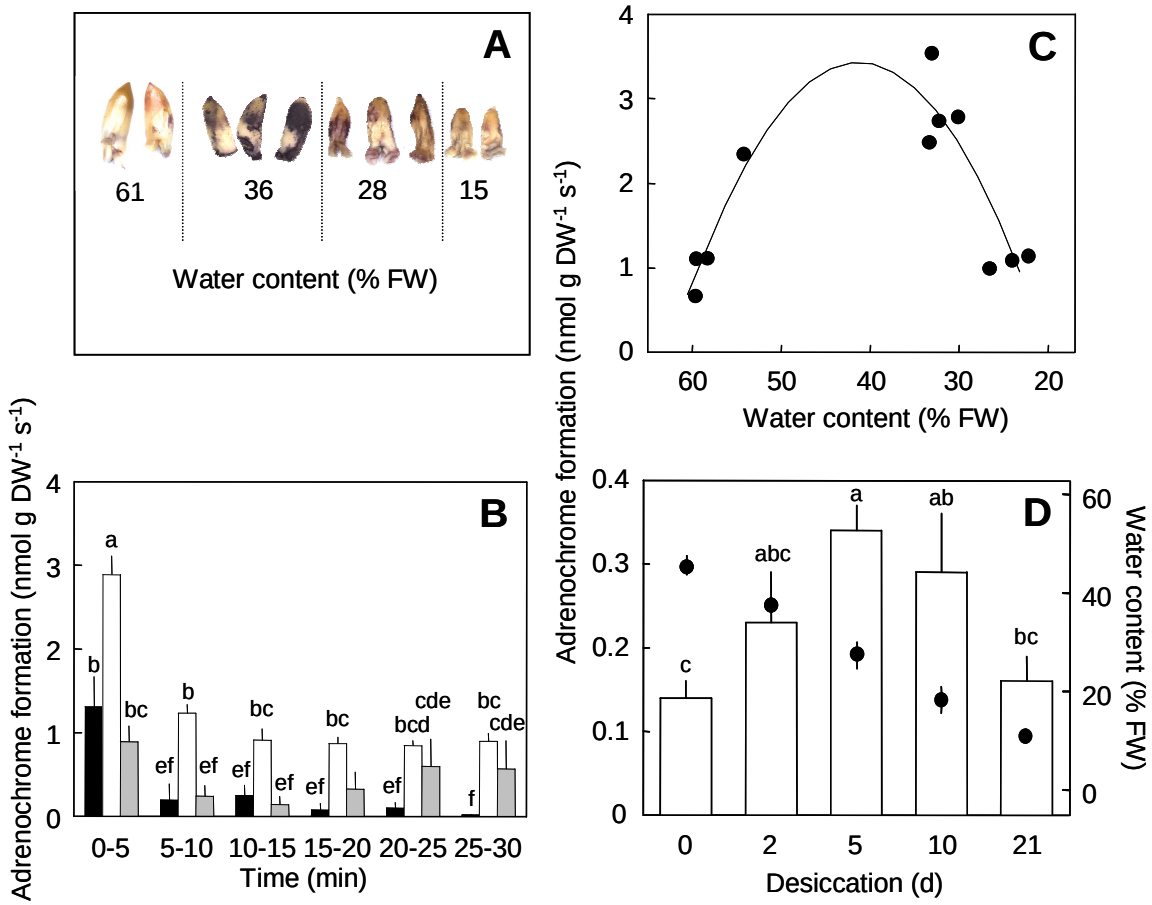


Figure 3.9 Effect of desiccation on extracellular $O_2^{\bullet -}$ production by *Castanea sativa* embryonic axes. **[A]** The location of superoxide production was visualised by incubating embryonic axes of various WCs in NBT solution, which turns dark purple in the presence of $O_2^{\bullet -}$. **[B]** Courtesy of Miss Mariyana Ivanova, $O_2^{\bullet -}$ production was quantified by measuring the oxidation of epinephrine to adrenochrome in 3.5 ml incubation solution after desiccating isolated embryonic axes from seeds, 2006, for 1 (black bars), 5 (white bars) and 10 h (grey bars). For each desiccation interval the same embryonic axes were re-used for every time interval. **[C]** The individual datum points of $O_2^{\bullet -}$ production from 0 – 5 min interval of [B] are expressed as WC. Each measurement is composed of five axes **[D]** The production of $O_2^{\bullet -}$ following the desiccation of embryonic axes within seed, 2007. Axes were rinsed after excision to remove cytosolic enzymes and incubated in 1.5 ml of epinephrine for 1 h, sufficient time to fully rehydrate the axis. Black symbols represent the WC following desiccation (right x axis) and bars shows the rate of $O_2^{\bullet -}$ production (left x axis). In [B] and [D] n = 4 replicates of 5 axes.

3.3 Discussion

3.3.1 Desiccation-induced changes in total germination, embryonic axis growth and viability markers

There are likely to be several reasons why *C. sativa* seeds, like other recalcitrant seeds, are intolerant to full desiccation (Pammenter and Berjak, 1999; Berjak and Pammenter, 2008), including structural complications at low WC. Here, isolated *C. sativa* embryonic axes displayed greater tolerance to desiccation than intact seeds (Fig. 3.1B; Fig. 3.1C). In 2006, embryonic axes were desiccated faster than seeds suggesting that desiccation rates influence critical WCs as discussed below. However, a comparison of total germination and isolated embryonic axis viability from identically desiccated seeds revealed that germination was far lower than embryonic axis viability (Fig. 3.2B). Failure of desiccation-stressed seeds to germinate containing viable embryonic axes could be due to several reasons. Due to their high saccharide content (Steadman *et al*., 1996), *C. sativa* seeds are prone to infection, as observed in Fig. 3.3A. Desiccation-induced losses in germination vigour could enable pathogens an opportunity to critically infect the seeds before defence mechanisms during germination become operational. Alternatively, the cotyledons may have suffered desiccation-related mechanical problems retarding germination. *Castanea sativa* embryonic axes may be better prepared for desiccation than the cotyledon as axes contain 46 and 180 kD dehydrins that could facilitate macromolecular stability (Farrant *et al*., 1996). *Castanea sativa* embryonic axes are recoverable to healthy plants down to WCs of 20 % (Pence, 1992; Corredoira *at al*., 2004). Survivability down to this WC suggests that macromolecular structures are sufficiently maintained or repaired at the WCs *C. sativa* axes were desiccated to here (Fig. 3.1A; Fig. 3.1B), indicating that other stresses, such as disrupted metabolism, at higher hydration levels caused viability loss in embryonic axes (Vertucci and Farrant, 1995).

Many studies show that faster desiccation rates permit seed and axis survival to lower WCs than when seeds or axes are desiccated more slowly [e.g. *Avicennia marina* (Berjak *et al*., 1984); *Hevea brasiliensis* (Normah *et al*., 1986); *Quercus rubra* (Pritchard, 1991) and *robur* (Pritchard and Manger, 1998); *Trichilia dregeana* (Kioko *et al*., 1998); *Ekebergia capensis* (Pammenter *et al*., 1998); *Wasabia spp.* (Potts and Lumpkin, 2000) and *Telfairia occidentalis* (Ajayi *et al*., 2006)]. Survival related to desiccation rate is in line with the hypothesis that disrupted metabolism during desiccation is responsible for viability loss (Walters *et al*., 2001). Recalcitrant seeds require high WC and exhibit continual metabolism from seed maturation on the maternal plant to germination. They are ill-prepared for low WCs and they can not shutdown metabolism (Franks and Drake, 2003). Hence, an enhanced formation of intracellular ROS occurs during recalcitrant seed desiccation, which has previously been shown to initiate an increase in antioxidant enzyme activities (Pukacka and Ratajczak, 2006; Cheng and Song, 2008). Desiccation-induced ROS production can exceed the capacity of the antioxidant defences and as a consequence oxidative stress prevails and seed viability declines (Finch-Savage *et al*., 1994; Francini *et al*., 2006; Kranner *et al*., 2006).

Oxidative stress from desiccation-induced ROS production can initiate lipid peroxidation events causing critical membrane damage (Senaratna *et al*., 1987; Varghese and Naithani, 2002). Rapidly desiccating isolated *C. sativa* embryonic axes to a WC of 25 %, corresponding with the onset of viability loss (Fig. 3.1), significantly increased electrolyte leakage (Fig. 3.4), a classic indicator of membrane integrity (ISTA, 1995). Leprince *et al*. (2000) showed that desiccation-induced metabolic dysfunction in recalcitrant seeds, suggestive of enhanced ROS production, occurred before a loss of membrane permeability. Therefore, it can be inferred that axes with severely leaky membranes would have endured some membrane-associated oxidative stress. The $E_{GSSG/2GSH}$ shift of +34 mV within embryonic axes desiccated to a similar WC is also in agreement with this (Fig. 3.6A).

Glutathione is an essential molecule that mitigates oxidative stress from ageing processes (Creissen *et al.*, 1999; Apel and Hirt, 2004). A study into moist storage of *C. sativa* seeds at 5 ^{o}C showed depletion of GSH reserves within 10 months while seedling quality fell (Greggains *et al.*, 2000). The concentrations of GSH and GSSG are used to calculate $E_{GSSG/2GSH}$ that can provide a reliable marker to seed viability (Kranner *et al.*, 2006). Here, *C. sativa* seeds desiccated for 5 d expressed an increase in $E_{GSSG/2GSH}$ of 12 and 13 mV to -207 and -211 mV for the axis and cotyledon, respectively (Fig. 3.6A), suggesting that cells had suffered only mild oxidative stress. However, 5 d desiccated seeds failed to germinate despite *c.* 90 % of isolated embryonic axes retaining viability (Fig. 3.2B). The correlation between the viability and $E_{GSSG/2GSH}$ of the embryonic axis was tight (R^2 = 0.916), but only a weak connection between axis $E_{GSSG/2GSH}$ and total germination (R^2 = 0.445) was observed (Table 3.1). This indicates that when isolated, embryonic axis viability was influenced by the oxidation state of glutathione, where as within seed other factors are more influential to germination.

In non-photosynthetic tissue, respiration is a major source of intracellular ROS, particularly during stress (Chance *at al.*, 1979; Moller, 2001). A healthy embryonic axis consumes around eight times the amount of $^{3}O_2$ as the cotyledon (Leprince *et al.*, 1999), indicating that an embryonic axis has the potential to suffer disrupted respiration to a greater extent than the cotyledon. The greater accumulation of disulphides in the embryonic axis (Fig. 3.5), which led to a bigger change in the $E_{GSSG/2GSH}$ (Fig 3.7A), suggests that the embryonic axes had suffered oxidative stress to a greater degree than the cotyledons. Oxidised glutathione is recycled back to GSH by glutathione reductase (GR), and slight increases in GR have been observed in other desiccation-stressed recalcitrant seeds (Pukacka and Ratajczak, 2006; Cheng and Song, 2008). This indicates recalcitrant seed defence mechanisms may be up-regulated in response to desiccation, but here it is clearly shown they have limits due to the accumulation of disulphides (Fig. 3.5).

The change in the redox pools of glutathione during desiccation-induced viability losses are consistent with previous studies showing the increased oxidation of glutathione during ageing of orthodox seeds (Kranner and Birtić, 2005; Kranner *et al*., 2006). After glutathione, the second most dominant LMW thiol – disulphide redox pair in the embryonic axis and cotyledon was cyst(e)ine (Fig. 3.5). The rapid loss of cysteine following 5 d of desiccation (Fig. 3.5) suggests that the amino acid is used in the initial stages against desiccation stress. Possibly, cysteine can protect thiol-containing proteins from irreversible oxidation, similar to the role of glutathione in glutathionylation (Kranner and Grill, 1996). Alternatively, GSH synthesis became induced, which rapidly diminished the pool of cysteine that has previously been shown to have occurred in response to numerous stresses (Vanacker *et al*., 2000; Noctor *et al*., 2002). However, only very small amounts of γ-glutamyl-cysteine (*c.* 300 times lower than GSH), the intermediate building block of GSH were observed (Fig. 3.5). Cysteinyl-glycine, the other dipeptide that was detected, was also found at almost as low concentrations as γ-glutamyl-cysteine, and therefore having a very limited effect on the cellular redox homeostasis. Because of their less negative standard redox potentials and lower concentrations the $E_{Cys/2Cys}$, $E_{glucys/2glucys}$ and $E_{cysgly/2cysgly}$ were all less negative in non-desiccated seed than $E_{GSSG/2GSH}$ (Fig. 3.6). A less negative $E_{CysS/2Cys}$ to $E_{GSSG/2GSH}$ has also been found in human plasma (Jones *et al*., 2000). However, it should be noted that at the same ratio of thiols to disulphides, redox pairs at higher concentrations naturally have lower half cell reduction potentials (Schafer and Buettner, 2001). Overall, all four LMW thiol – disulphide redox couples suggest an enhanced production of cellular ROS occurs during desiccation, with a dominant role for GSH and a contribution by cysteine in mitigating oxidative stress. Although, ROS can cause oxidative stress, they can also be important components to stress-signalling and plant physiology. The wounding-induced oxidative burst is discussed next.

3.3.2 Isolation of embryonic axes causes a burst of superoxide in response to wounding

A burst of ROS is a typical response to wounding as has been shown for wheat roots (Minibayeva *et al.*, 2009), tomato leaves (Orozco-Cardenas and Ryan, 1999), seaweed (Ross *et al.*, 2006), squash mesocarp (Watanabe and Sakai, 1998) and lichens (Beckett *et al.*, 2004), but so far has not been characterized following the isolation of embryonic axes from seeds. Results here show that axis excision from non-desiccated seeds causes a rapid burst of $O_2^{\bullet-}$ within the first 5 min, as demonstrated by colourimetric detection of $O_2^{\bullet-}$ by epinephrine oxidation, an ESR spectrum of the Tiron semiquinone radical typically produced by the reaction of Tiron with $O_2^{\bullet-}$ and by NBT staining (Fig. 3.8). The detection of $O_2^{\bullet-}$ in the incubation medium suggests that $O_2^{\bullet-}$ was produced extracellularly, as has been reported elsewhere as a typical stress response (Minibayeva *et al.*, 1998; de Bruxelles and Roberts, 2001; Leon *et al.*, 2001). It has been suggested that ROS serve as a first line of defence against potential pathogen attack (Doke, 1985; Bolwell *et al.*, 1995). Wounding also causes a release of cell wall oligosaccharides (Leon *et al.*, 2001), which are well-characterized elicitors of defence responses (Côté and Hahn, 1994) that include an oxidative burst (Stennis *et al.*, 1998).

Data in Figure 3.9B shows the rate of $O_2^{\bullet-}$ production using an experimental design in which the incubation solution was changed every 5 min. To examine the patterns of $O_2^{\bullet-}$ production further, new embryonic axes were used for each incubation that had been kept hydrated for up to 8 h (Fig. 3.8C). Using new axes for measurements 25 – 30 min after excision, $O_2^{\bullet-}$ production was about four times higher than in axes that had been used for previous measurements (Fig. 3.8). This suggests that enzymes or substrates involved in $O_2^{\bullet-}$ production are loosely bound to the cell wall or transiently released, and partly lost by changing the incubation solution. The production of $O_2^{\bullet-}$ from the wounded surface was totally diminished 8 h after excision. It is possible that signalling molecules initiating activity were rapidly metabolised and substrates required for producing $O_2^{\bullet-}$, such as reductants (Halliwell and

Gutteridge, 1999), became depleted. The overall decrease in $O_2^{\bullet-}$ production over time suggests the presence of $O_2^{\bullet-}$ is only necessary for a limited length following excision, reflecting the transient nature of the oxidative burst.

Previous studies showed that extracellular ROS production limits the spread of invading pathogens. Superoxide concentrations that are lethal to pathogens are difficult to estimate due to the short half-life of $O_2^{\bullet-}$ (10 µsec), but experiments using H_2O_2, which is more stable, suggest that 5 to 20 µM H_2O_2 can inhibit the germination of the spores of pathogenic fungi, and 0.05 to 0.10 nM H_2O_2 can inhibit bacterial growth (*citations in* Murphy *et al.*, 1998). In Fig. 3.8A, about 80 mg of plant material, the FW of 5 embryonic axes, produced $O_2^{\bullet-}$ concentrations of 10 µM during 5 min after wounding in 3.5 ml of incubation solution. Assuming that all $O_2^{\bullet-}$ dismutates to H_2O_2, this would give a concentration of 5 µM H_2O_2, sufficiently high to inhibit fungal spore germination and bacterial growth, but $O_2^{\bullet-}$ itself will also be directly toxic irrespective of dismutation to H_2O_2. However, while effective $O_2^{\bullet-}$ concentrations of 10 µM were found in the large volume of incubation solution used in our assay, a considerably greater concentration of $O_2^{\bullet-}$ would be present on the actual cut surface of an excised axis. Therefore, the initial oxidative burst following axis excision could serve as an efficient defence strategy against microbial pathogens, preventing penetration through the wound. Furthermore, the presence of H_2O_2 from $O_2^{\bullet-}$ dismutation could also participate in cell wall restructuring and reinforcing cellular defences (Bradley *et al.*, 1992; Otte and Barz, 1996) preventing pathogen entry.

3.3.3 Desiccation induces a bell-shaped response of superoxide production

Superoxide production was initially confined to the cut surface immediately following embryonic axis isolation from non-desiccated seeds (Fig. 3.8A), but the production of $O_2^{\bullet-}$ spread around the axis surface following desiccation to intermediate WC (Fig. 3.9A). Desiccating excised embryonic axes from a WC of *c.* 60 % to *c.* 30 % doubled the initial rates of extracellular $O_2^{\bullet-}$ production

(Fig. 3.9B). Further desiccation, severely stressing embryonic axes as demonstrated by loss of more than half of their vigour and viability (Fig. 3.1C), lowered the rate of $O_2^{\bullet-}$ production. Reductions in $O_2^{\bullet-}$ production could be due to the inactivation of redox enzymes responsible for $O_2^{\bullet-}$production and/or the deficiency of substrates, such as reductants. Considering the oxidative environment of severely desiccation-stressed axes, as indicated by the lowered concentration of LMW thiols (Fig. 3.5), the abundance of reductants, putatively used in enzymatic extracellular ROS production (Halliwell *et al*., 1978; Schopfer *et al*., 2008), is likely to be scarce.

Desiccating embryonic axes at a slower rate within seeds displayed a similar pattern of extracellular $O_2^{\bullet-}$ production, also showing maximal rates at *c.* 30 % WC (Fig. 3.2A; Fig. 3.9D). Desiccation first increased the rate of $O_2^{\bullet-}$ production while axis viability was maintained, whereas prolonged desiccation for 10 d and 21 d decreased viability and production of $O_2^{\bullet-}$. It is possible that imbibitional stress was a cause of some $O_2^{\bullet-}$ production by the nature of epinephrine measurements. However, imbibing desiccated embryonic axes for 1 h rather than 5 min while taking measurements of $O_2^{\bullet-}$ reduced the rates of $O_2^{\bullet-}$ production in axes, which had become more rehydrated, suggesting that imbibitional stress was minimal (Fig. 9B; Fig. 9D). Overall, regardless of how embryonic axes were desiccated, a bell-shaped relationship between WC and $O_2^{\bullet-}$ production was observed, typical of a stress response.

3.3.4 Summary

This chapter provides further insights into desiccation-induced viability loss of *C. sativa* seeds and how embryonic axes respond to excision and during desiccation. Using several markers of viability has highlighted some important insights into desiccation-related death. Total germination was shown to be unreliable as an overall viability marker, but the embryonic axis $E_{GSSG/GSH}$ and isolated axis viability correlated very well. Separate measurements of embryonic axes and cotyledons highlighted some intriguing differences in oxidative stress between the two organs. Initially, the embryonic axis had

higher concentrations of LMW thiols, suggesting that axes are better protected by antioxidants, but these became unbalanced during desiccation and the embryonic axis suffered greater oxidative stress than the cotyledons.

Excising embryonic axes from *C. sativa* seeds was accompanied by a sharp and transient increase in extracellular ROS production. Three different assays provided evidence for the involvement of $O_2^{\bullet -}$ as a major component of the oxidative burst. Mild desiccation led to increases in extracellular $O_2^{\bullet -}$ production, suggesting that extracellular $O_2^{\bullet -}$ formation possibly serves as a stress-signal of the oxidative damage occurring internally. The desiccation-induced oxidation of LMW thiols, which occurred before viability loss. Further desiccation below 30 % WC, associated with viability loss, led to a decline in extracellular $O_2^{\bullet -}$ production. The $E_{GSSG/2GSH}$ could accurately assess embryonic axis viability, whereas germination was unreliable. Oxidative stress-associated viability loss would occur through irreversible damage to, for example, phopholipid membranes as observed at *c.* 25 % WC. The pleiotropic pattern of extracellular $O_2^{\bullet -}$ production reflects both the adaptive and the detrimental stages of the responses of organisms to stress in general. Major future challenges in the improvement of cryopreservation protocols for recalcitrant seeds are likely to involve the modulation ROS production, which is explored in the next chapter along with the identifying the mechanisms of enzymatic ROS production.

Chapter 4 Identifying the mechanisms of extracellular superoxide production by *Castanea sativa* embryonic axes and the effects of modulating reactive oxygen specie activity

4.1 Introduction

In chapter 3, it was described that desiccation of *Castanea sativa* embryonic axes to between 40 and 30 % WC led to enhanced extracellular ROS production (Fig. 3.9). It was suggested that extracellular ROS could be beneficial by serving as a stress signal. Following excision, wounding also initiated a burst of $O_2^{\bullet-}$, postulated to be sufficiently concentrated to sterilise the cut surface and help repair damaged cell walls. However, the mechanisms of extracellular ROS production by *C. sativa* are currently unknown. Measurements of $O_2^{\bullet-}$ were taken of the incubation solution confirming that production was extracellular. Proteins make up 10 % or less of the cell wall mass (Fry, 1988). There are several hundred different types of cell wall proteins that serve roles such as enhancing the structure of the polysaccharides matrixes and facilitate defence mechanisms (Cassab, 1998; Carpita *et al*., 2001). Recent proteomic studies have identified 43 and *c.* 200 extracellular enzymes in Arabidopsis and maize roots, respectively (Chivasa *et al*., 2002; Zhu *et al*., 2006). Borderies *et al*., (2003) found that a quarter of extracellular proteins are associated with defence, including ROS metabolising enzymes, such as ECPOX.

The flexibility of ECPOX activity has been well documented. The most well characterised ECPOX reaction involves the reduction of H_2O_2 (Passardi *et al*., 2005), but accumulating evidence suggests they also have $O_2^{\bullet-}$-producing capabilities (Hiner *et al*., 2001; Kawano, 2003; Mika *et al*., 2004; Minibayeva *et al*., 2009). ECPOX can be bound to the membrane (Mika and Lüthje, 2003), bound to the cell wall with ionic or covalent bonds (Rouet *et* al., 2006; Kukavica *et al*., 2009) or somehow released, through unknown mechanisms,

into the apoplast (Sgherri *et al*., 2007; Minibayeva *et al*., 2003; 2009). Major secretions of ECPOX and increases in ECPOX activity have been observed at plant infection sites (Bestwick *et al*., 1997; McLusky *et al*., 1999), the same locations where localized $O_2^{\bullet -}$ production occurs following pathogen challenges (Thordal-Christensen *et al*., 1997). In fact, transgenic Arabidopsis plants with lowered ECPOX expression had an impaired oxidative burst and increased susceptibility to bacterial and fungal challenges (Bindschedler *et al*., 2006). Thus, ECPOX are intricately involved in pathogen resistance, but are also likely to have many other roles in abiotic stress response due to their ability to metabolise ROS.

Any enzyme producing $O_2^{\bullet -}$ requires the availability of suitable electron donors. The most well characterised $O_2^{\bullet -}$-synthase enzyme is membrane bound NAD(P)H oxidase, the plant analogue of the enzyme from mammalian neutrophils. In plants, NAD(P)H oxidase has unique G-protein and calcium binding domains that regulate its activity (Lamb and Dixon, 1997; Keller *et al*., 1998). As NAD(P)H oxidase traverses the plasma membrane it can use cytosolic supplies of NAD(P)H to produce apoplastic ROS. However, enzymes without contact to the cytosol require reductants within the apoplast. Analysis of the apoplast during the elicitor induced ECPOX mediated oxidative burst of French bean cell cultures revealed no obvious reductant candidates (Bolwell *et al*., 1999). Phenolic acids, used for strengthening the cell wall by their conversion to lignin, could be potential reductants as they can donate electrons to ECPOX, as shown in Figure 4.1 below. Phenols have also been considered antioxidants because of their ability to quench free radicals (Graf, 1992). However, in the presence of transition metals (Sakihama *et al*., 2002) or H_2O_2 (Chasov and Minibayeva, 2009), phenolic acids and polymerized phenols can also be pro-oxidants (Takahama, 2004). During lignification ECPOX bind together phenolic acids via the creation of phenoxyl radical intermediates (Ros-Barcelo *et al*., 2004; Encina and Fry, 2005). If phenoxyl radicals can reduce $^3O_2$ to $O_2^{\bullet -}$, as has been suggested by Takahama (2004),

ECPOX may be able to use phenolic acids to produce $O_2^{\bullet -}$ in the presence of H_2O_2 (Fig. 4.1).

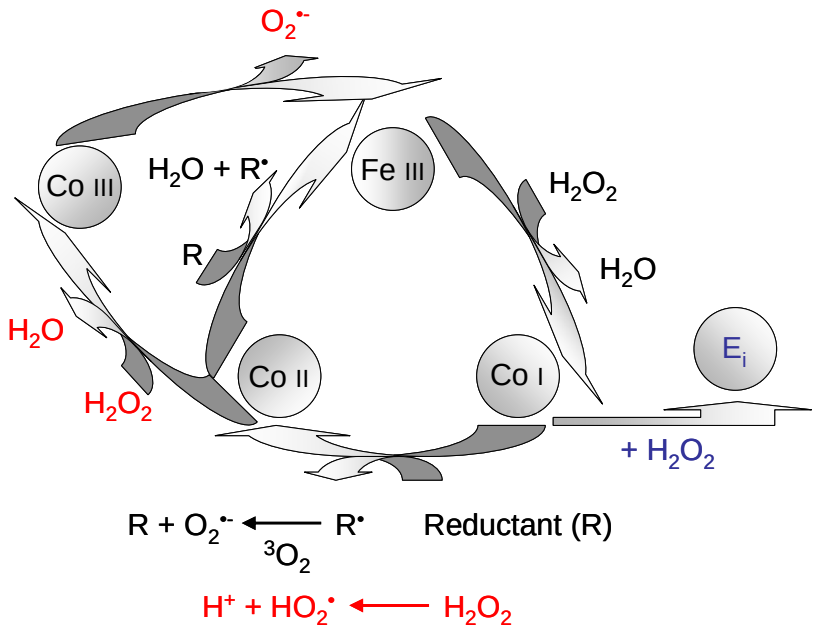


Figure 4.1 Simplified scheme of H_2O_2 scavenging and $O_2^{\bullet -}$ production by ECPOX. Scavenging of H_2O_2 involves oxidation of the ferric heme group^{+3} (FeIII) of ECPOX by donating two electons to H_2O_2 to form compound I^{+5} (CoI) and H_2O. Through two single electron reductions, the ECPOX oxidises substrates, typically phenolic compounds during lignification, by recycling through compound II^{+4} (CoII) back to the native form (FeIII). If the reduction potentials permit, reducant radicals ($R^{\bullet}$) produced can reduce $^3O_2$ to $O_2^{\bullet -}$ (Halliwell, 1978). In high concentrations of H_2O_2, as shown in red and blue text, the POX can be inactivated (verdohemoprotein, E_i) or form compound III^{+6} (CoIII) by a double electron reduction of H_2O_2, with ECPOX recycling back to the native form after emitting $O_2^{\bullet -}$ (Hiner *et al.*, 2001).

The enzymatic production of ROS could be for transducing cellular signals (Neill *et al.*, 2002; Apel and Hirt, 2004). The biochemical nature of certain ROS makes them excellent secondary messengers (Fedoroff, 2006; Foyer and Noctor, 2005; 2009). For example, ROS can be rapidly produced in a localised area of the apoplast. Hydrogen peroxide, either produced directly or through $O_2^{\bullet -}$ dismutation, can then migrate across the plasma membrane into the cytosol (Chance *et al.*, 1979; Henzle and Steudle, 2000). The cytosol is kept at a reduced redox state by antioxidants such as ascorbate and

glutathione (Foyer and Noctor, 2005), facilitating the contrast to a burst of H_2O_2. Here, H_2O_2 can target cysteine domains on proteins that are regulated through redox-induced reversible conformation changes (Rhee, 2006). The H_2O_2 signal can then be removed enzymatically or quenched by antioxidants, providing a precise control over redox activity and preventing damage by uncontrolled oxidation (Mika *et al.*, 2004). While localised and controlled production of ROS is integral to signalling networks involved in growth (Foreman *et al.*, 2003) and development (Gapper and Dolan, 2006), rapid and continued production can also serve to initiate programmed cell death (Raff 1998; Hengartner, 2000; Gechev *et al.*, 2006). Nevertheless, the role of extracellular ROS production in stress signalling by recalcitrant seeds has been given hardly any consideration.

Contrary to the benefits of ROS, if stress-induced production of ROS from disrupted metabolism can not be buffered by antioxidants, uncontrolled and irreversible oxidations occur. A typical reaction product of oxidative stress is carbonylation, causing inactivation, crosslinking or breakdown of intracellular proteins (Dalle-Donne *et al.*, 2003; Davies, 2004). In chapter 3, seed desiccation led to lowered vigour and viability (Fig 3.2) and was associated with an increasing accumulation of disulphides (Fig 3.5), indicative of oxidative stress. However, protein carbonylation has also been implicated with promoting germination in orthodox seeds (Oracz *et al.*, 2009), reflecting the dual nature of ROS activity and the complexities of unravelling damage versus signalling. Possibly linking carbonylation and germination, elevated rates of ROS production have been observed during seed imbibition and germination (Boveris, 1984; Schopfer *et al.*, 2001), but not if imbibing seeds are dormant (Schopfer *et al.*, 2001; Bailly *et al.*, 2008).

Previous experiments manipulating the abundance of ROS have led to some revealing insights into the roles of ROS for seed and seedling physiology. Treating orthodox seeds with H_2O_2 or inhibiting catalase activity can break physiological based dormancy (Hendricks and Taylorson, 1975; Fontaine *et al.*, 1994; Naredo *et al.*, 1998; Ogawa and Iwabuchi, 2001; Serath

et al., 2007; Oracz *et al*., 2009). Following germination, young Arabidopsis roots showed regional elevations of $O_2^{\bullet-}$ in the differentiation zone, that if scavenged, inhibited root growth (Dunand *et al*., 2007). Roots require $O_2^{\bullet-}$ production by NADPH oxidase to activate Ca^{+2} channels that maintain growth-dependent high Ca^{+2} concentrations in root tips (Foreman *et al*., 2003). Hydrogen peroxide has also been shown to be essential for the expansion of maize leaves (Rodriquez *et al*., 2002) and tobacco protoplast division (de Marco and Roubelakis-Angelakis, 1996). Thus, ROS seem to be integral signals co-ordinating different developmental stages between germination and seed death and requiring tight control to prevent undesired damage and stress. Considering that both excising and desiccating *C. sativa* embryonic axes led to increases in extracellular $O_2^{\bullet-}$ production, modulating this activity may lead to enhanced survival of desiccation-stressed recalcitrant seeds.

Here, to first understand the mechanisms of ROS production, the potential involvement of enzymes producing extracellular $O_2^{\bullet-}$ was tested using DPI, an inhibitor of NAD(P)H oxidase (Henderson and Chappell, 1996) and NaN_3, an inhibitor of hemoproteins such as ECPOX (Liu *et al*., 2006). The localisation and intensity of ROS-producing enzymes within the embryonic axis were gained by ROS-sensitive stains. A cell wall fractionation was conducted to study the forces binding the $O_2^{\bullet-}$-producing enzymes to the cell wall. As ECPOX were identified to be a major contributor to extracellular $O_2^{\bullet-}$ production, the ability of ECPOX-containing cell wall fractions to produce $O_2^{\bullet-}$ was tested in the presence of H_2O_2, NADH and phenolic acids. Polyacrylamide gel electrophoresis was used in conjunction with in-gel staining to reveal the molecular mass, prosthetic group and substrate specificity of extracellular enzymes involved in $O_2^{\bullet-}$ production.

In chapter 3, it was hypothesised that the desiccation-induced extracellular $O_2^{\bullet-}$ production is a stress signal, which could potentially be manipulated, either through suppression or stimulation. Scavenging of excess ROS by exogenously applied antioxidants could help in ameliorating adverse effects of ROS. In contrast, stimulation of ROS production could invigorate

desiccation-stressed embryonic axes, which have compromised signalling capacities. The effects of applying various concentrations of an anti-oxidant (GSH) and a pro-oxidant (H_2O_2) on the growth of viable embryonic axes removed from seeds unable to germinate were observed. Finally, the development of embryonic axes was followed for 21 d of growth to see if root and shoot development is accompanied by the production of extracellular $O_2^{\bullet -}$.

4.2 Results

4.2.1 Reactive oxygen species production by the embryonic axis

In chapter 3 it was shown that excising embryonic axes initiates the immediate production of $O_2^{\bullet-}$ from the wounded surface (Fig. 3.8). Figure 4.2 suggests that the embryonic axis perimeter is particularly enriched with abilities to produce $O_2^{\bullet-}$ and its dismutation product H_2O_2. The xylem also stained strongly for both ROS with higher activity at the radicle tip than the shoot tip (Fig. 4.2). Incubation of excised embryonic axes from non-desiccated seeds with SOD significantly decreased adrenochrome formation by 60 % ($P < 0.05$, Table 4.1), revealing the specificity of the epinephrine assay to $O_2^{\bullet-}$. Incubation of excised axes with 1 or 10 µM DPI and 1 mM NaN_3 also decreased $O_2^{\bullet-}$ production by 30 % to 40 % ($P < 0.05$). Superoxide production was also recorded after removal of the axes from the incubation solution ('leachate control' in Table 4.1), suggesting that some of the enzymes capable of ROS formation and their substrates were released into the incubation medium. The leachates produced 80 % less $O_2^{\bullet-}$ in the presence of 0.5 mM NaN_3, suggesting that hemoproteins, such as ECPOX, were involved in $O_2^{\bullet-}$ production.

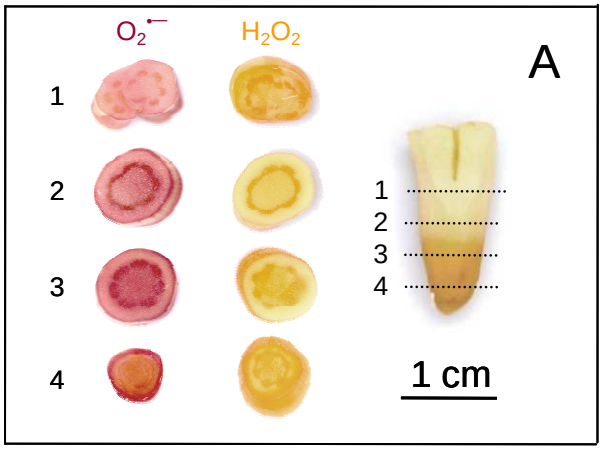


Figure 4.2 Localisation of ROS production in isolated embryonic axis of *Castanea sativa*. The right image shows an unstained axis and the dotted lines denote the area where cross sections (left images) were taken. The far left cross sections show $O_2^{\bullet-}$ production after staining with nitroblue tetrazolium (NBT; dark purple stain). The near left sections shows H_2O_2 production in conjunction with ECPOX activity after incubation in 3,3'-diaminobenzidine (DAB; dark yellow staining). Note the intense NBT staining on the outer edge of the cut surface.

Table 4.1 Effects of SOD and the enzyme inhibitors DPI and NaN_3 on $O_2^{\bullet-}$ production in leachates of excised *Castanea sativa* axes. Axes were excised and incubated with SOD or enzyme inhibitors for 10 min and $O_2^{\bullet-}$ concentrations measured after further incubation with epinephrine for 5 min. 'Leachate control' and 'leachate + NaN_3' show rates of $O_2^{\bullet-}$ production over 30 min in the leachates (without axes) in the absence or presence of NaN_3, respectively. Leachates were obtained by soaking excised axes in 2 ml of distilled water for 1 h. Data represent mean ± SE (n = 3 replicates of 5 axes each). Data labelled with the same letter do not differ significantly ($P < 0.05$).

Treatment	$O_2^{\bullet-}$ (% of control)
Control	100 [a]
SOD (250 units ml^{-1})	40 [c]
DPI (1 µM)	64 [b]
DPI (10 µM)	67 [b]
NaN_3 (0.1 mM)	97 [a]
NaN_3 (1 mM)	61 [bc]
Leachate control	100 [A]
Leachate + NaN_3 (0.5 mM)	21 [B]

4.2.2 Putative enzymes and reductants involved in extracellular superoxide production in cell wall fractions

To study cell wall enzymes that are potentially involved in extracellular $O_2^{\bullet-}$-production, cell wall fractions were measured for H_2O_2-scavenging activity, in the presence of ABTS, and their ability to produce $O_2^{\bullet-}$. In addition, the involvement of putative cell wall-bound reductants required for $O_2^{\bullet-}$production was studied (Fig. 4.3). In embryonic axes and cotyledons, 64 % and 28 %, respectively, of the H_2O_2-scavenging activity was found in the crude intra-cellular fraction (Fig. 4.3A). Enzyme activity in the crude cell fraction of embryonic axes was *c.* 15 times higher than in cotyledons. In isolated embryonic axes, the activity of H_2O_2-scavenging enzymes was also found in three cell wall fractions, 17 % of which were loosely bound to the

cell wall (B1), 11 % were bound by hydrophobic interactions (B2), and 72 % were ionically bound (B3). In cell wall fractions of cotyledons, activity of H_2O_2-scavenging enzymes, such as ECPOX, was only detected in fraction B3, displaying half of the activity in the B3 embryonic axis fraction. Almost all H_2O_2-scavenging enzymes could be removed form the cell walls with barely detectable activity remaining in the B4 fraction (Fig. 4.3A). Cytoplasmic contamination, assessed by measuring G6PDH activity, was found only in the B1 fraction at 2% of the G6PDH activity in the crude cell fraction.

The B3 cell wall fraction of embryonic axes produced $O_2^{\bullet-}$ at significantly higher rates than all other fractions, observed at around two and three times more than the crude cell fraction of the axes and the cotyledons, respectively (Fig. 4.3B). All other cell wall fractions of embryonic axes and cotyledons produced relatively little $O_2^{\bullet-}$; rates were below 10 nmol g^{-1} DW s^{-1}. In the experiments presented in Figure 4.3C, NADH was used as a "control" reductant. NADH stimulated $O_2^{\bullet-}$ production significantly with at a maximum concentration of 0.1 mM (Fig. 4.3C). However, the presence of NADH in the apoplast is questionable and so potential reductants available in the apoplast, such as phenolic acids (Sukalovic *et al.*, 2005; Kukavica *et al.*, 2009), were tested (Fig. 4.3D). Ferulic acid, a coniferyl alcohol precursor, stimulated $O_2^{\bullet-}$ production six times more than NADH at 0.1 mM. *p*-coumaric acid, a lignol precursor to ferulic acid, also increased $O_2^{\bullet-}$ production by 1.5 times more than NADH. Cinnamic acid had the least effect, but still stimulated the same rate of $O_2^{\bullet-}$ production as NADH at 0.1 mM. Interestingly, the hydroxycinnamic acids did not show any feedback inhibition at higher concentrations as was observed for NADH. In the absence of H_2O_2, rates of $O_2^{\bullet-}$ production were considerably reduced (Fig. 4.3C), suggesting that extracellular $O_2^{\bullet-}$ production strongly depended on the availability of H_2O_2.

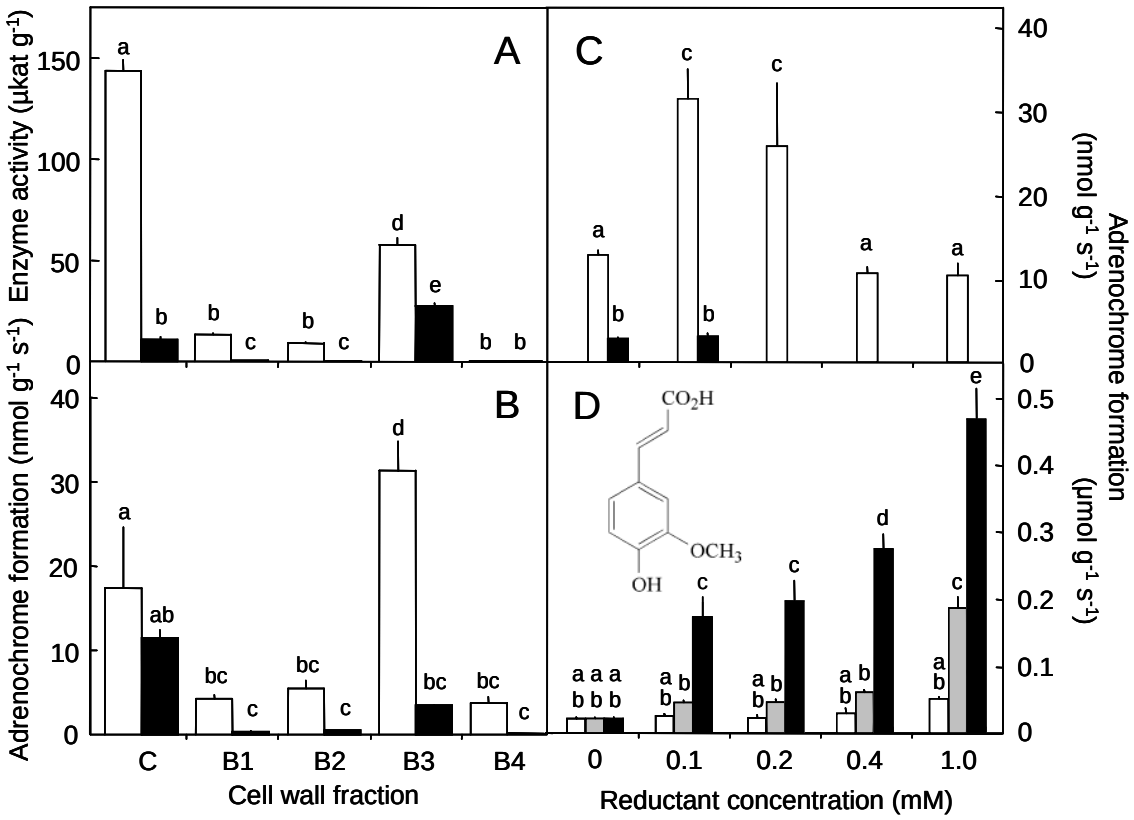


Figure 4.3 Activity of H_2O_2-degrading enzymes and $O_2^{\bullet-}$-production in various cellular fractions of *Castanea sativa* embryonic axes and cotyledons. Cell fractions were; C, cytosolic fraction; B1, proteins loosely associated to the cell wall; B2, cell wall proteins bound by hydrophobic interactions; B3, cell wall proteins bound by ionic bonds; B4, irremovable cell wall proteins. **[A]** Activity of enzymes within the cell wall fractions that catalyze the H_2O_2-dependent oxidation of ABTS, such as ECPOX. **[B]** Superoxide production, as indicated by adrenochrome formation, by cell wall fractions in the presence of 0.1 mM NADH and 10 mM H_2O_2, quantified spectrophotometrically after incubation with epinephrine in 1.5 ml reaction volume. In [A] and [B], white and black bars represent embryonic axes and cotyledons, respectively. **[C]** Superoxide production in fraction B3 in the presence of various concentrations of NADH and 10 mM H_2O_2 (white bars). Measurements without H_2O_2 were made with NADH concentrations 0 and 0.1 mM (black bars). **[D]** Superoxide production in fraction B3 in the presence of various concentrations of cinnamic acid (white bars), *p*-coumaric acid (grey bars) and ferulic acid (black bars) with 10 mM H_2O_2. Data shows mean + SE (n = 4). The inset shows the structure of ferulic acid.

To further investigate the nature of ionically bound $O_2^{\bullet-}$-producing enzymes in the embryonic axis, the B3 cell wall fraction was electrophoretically separated and studied using in-gel staining. Results suggest the presence of a 44 kDa ECPOX unable to produce $O_2^{\bullet-}$ in the conditions provided. However, a 63 kDa protein was capable of oxidising NADH with or without H_2O_2, and produce $O_2^{\bullet-}$ with ferulic acid, but only in the presence of H_2O_2 (Fig. 4.4).

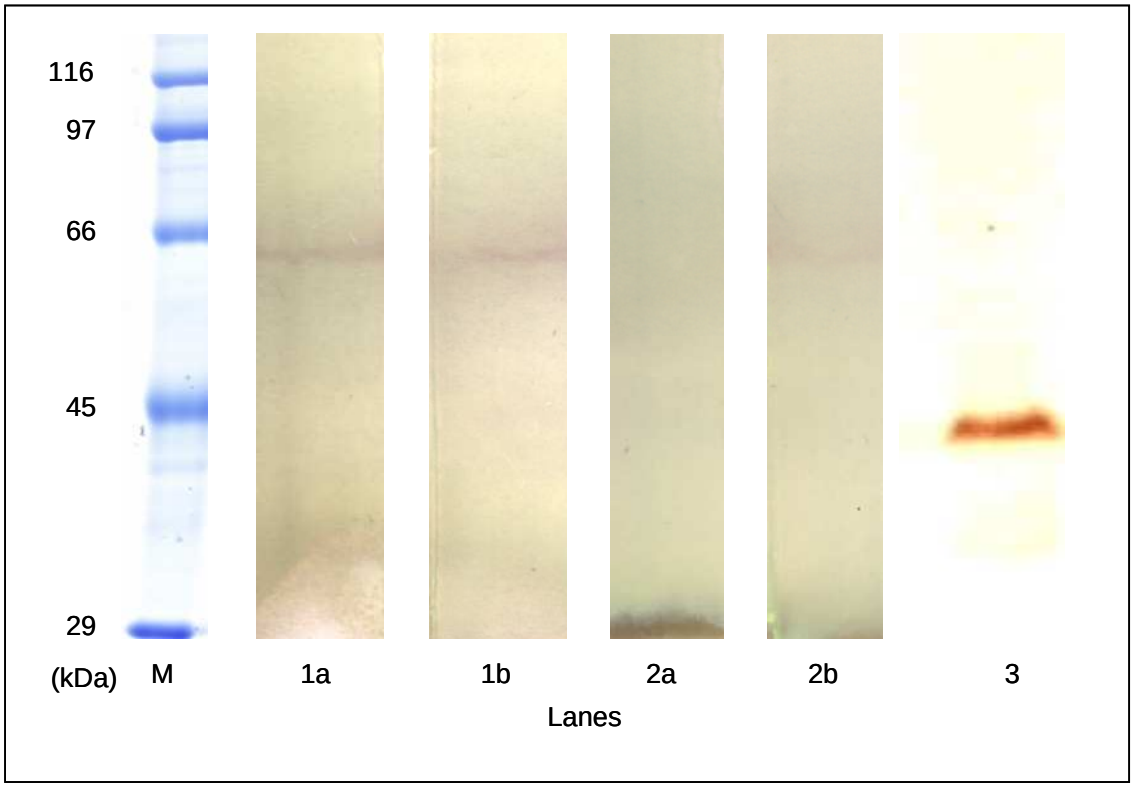


Figure 4.4 Enzyme activity stains following electrophoretic separation of ionically bound cell wall proteins of *Castanea sativa* embryonic axes. From left to right: Lane (M); the molecular mass of the marker (kDa). Lanes (1) and (2) indicate $O_2^{\bullet-}$ production by the formation of purple bands following incubation in NBT and 0.1 mM NADH (lanes 1) or 0.1 mM ferulic acid (lanes 2), in the absence (lanes a) or presence (lanes b) of 10 mM H_2O_2. A $O_2^{\bullet-}$-producing protein of *c.* 63 kDa was identified in the presence of NADH regardless of the availability of H_2O_2, but ferulic acid was only capable of producing $O_2^{\bullet-}$ production in the presence of H_2O_2. Lane 3 shows a *c.* 44 kDa protein stained with guaiacol and 10 mM H_2O_2, indicative of a ECPOX, that was unable to produce $O_2^{\bullet-}$ with either NADH or ferulic acid.

4.2.3 Effects of hydrogen peroxide on seed germination and on the vigour of isolated embryonic axes

Treatment of non-desiccated seeds with H_2O_2 for 1 h had a concentration-dependent effect upon subsequent germination. Concentrations of 0.1 M and 0.5 M H_2O_2 significantly ($P < 0.05$) increased total germination by 22 %, but 1.0 M H_2O_2 had no statistically significant effect (data not shown). Seeds soaked in 1.0 M H_2O_2 bubbled vigorously (Fig. 4.5A) suggestive that H_2O_2 was rapidly broken down to $^3O_2$. Soaking non-desiccated seeds in either water or 1.0 M H_2O_2 increased WC, vigour and total germination compared to non-soaked seeds (Fig. 4.5B). No statistical differences were found between the H_2O or 1.0 M H_2O_2 treatments, indicating that the water taken up rather than H_2O_2 had enhanced seed vigour.

Mild desiccation for 2 d decreased total germination of un-treated seeds by 18 %. Soaking these mildly desiccated seeds in H_2O improved total germination by 15 %, whereas incubating them in 1.0 M H_2O_2 promoted total germination by 40 %, significantly higher ($P < 0.05$) than soaking in H_2O (Fig. 4.5C). After 5 d of desiccation the seeds reached a critical WC of 28 ± 3 % and failed to germinate. In these lethally stressed seeds, soaking in neither H_2O nor in H_2O_2 enhanced viability (Fig. 4.5D). However, 87 ± 4 % of embryonic axes removed from 5 d desiccated seeds (WC of 31 ± 4 %) were still viable, 53 ± 4 % growing shoots after 21 d in tissue culture. Snapshots of a typical viable embryonic axis over 21 d are shown in Figure 4.6A, with shoot formation starting after 15 d in tissue culture. During the first 10 d of growth of embryonic axes excised from 5 d desiccated seed, axes only elongated without gaining DW, but increases in extracellular $O_2^{\bullet-}$ production occurred (Fig. 4.6B). Embryonic axes in tissue culture only started gaining DW between 10 d and 15 d, coinciding with falling rates of $O_2^{\bullet-}$ production.

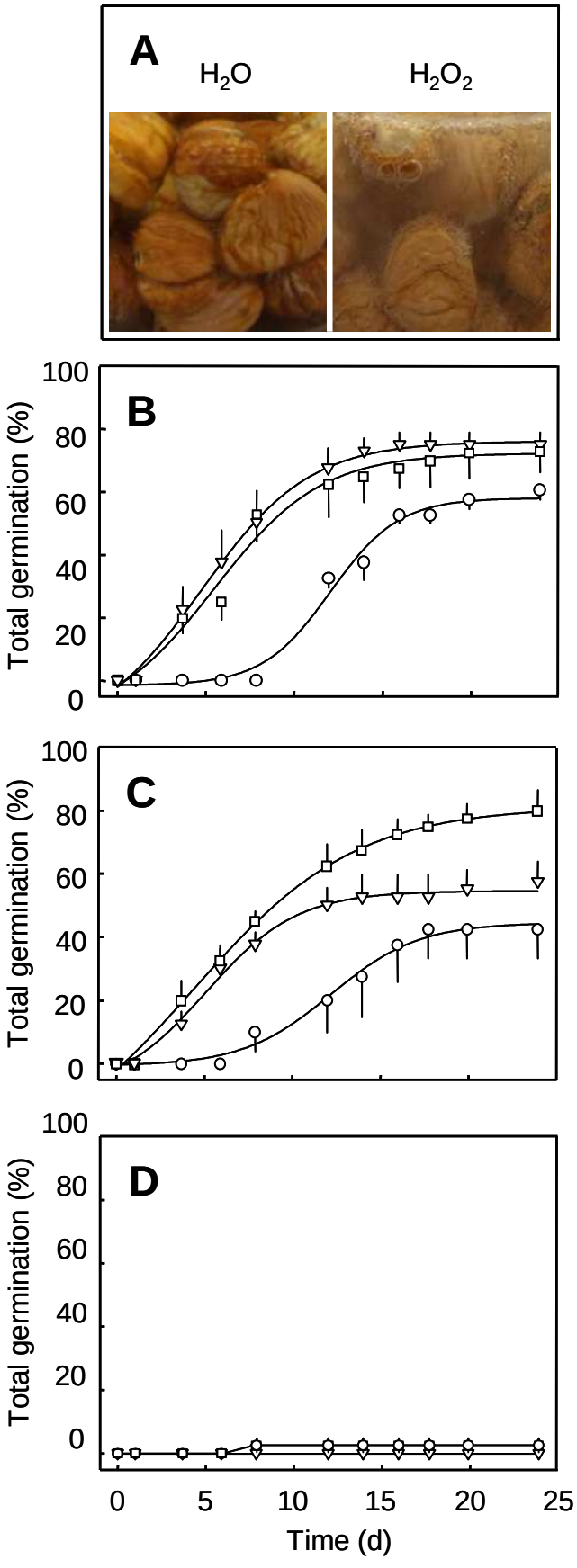


Figure 4.5 Effects of pre-sowing applications of H_2O and H_2O_2 on the germination of *Castanea sativa* seeds at various WC. **[A]** The production of $^3O_2$ (note the gas bubbles) of non-desiccated *C. sativa* seeds incubated in 1.0 M H_2O_2 (right), compared to H_2O (left). **[B]**, **[C]** and **[D]** show total germination of seeds desiccated at 15 % RH and 15 ^OC for 0, 2 and 5 d, respectively. Desiccation for 0, 2 and 5 d corresponded to seed WCs of 45 ± 2 %, 38 ± 2 % and 27 ± 1 %, respectively and soaking these seeds for 1 h increased the WC to 48 ± 1 %, 43 ± 1 % and 32 ± 1 %, respectively. Circles denote untreated [B] or desiccated [C, D] seeds. Triangles denote seeds that were soaked for 1 h in distilled H_2O and squares denote seeds that were treated with 1 M H_2O_2 for 1 h (means ± SE, n = 5 replicates of 8 seeds).

Treating axes isolated from 5 d desiccated seeds with 0.1 mM H_2O_2 significantly ($P < 0.05$) enhanced viability by 17 %, expressed as a percentage of axes that grew roots compared to H_2O-treated control (Fig. 4.6C), and vigour, expressed as root elongation, which increased by 0.2 mm per day more than that of controls (Fig. 4.6D). The gains in viability were lost by treating axes with concentrations above 0.1 mM H_2O_2 (Fig. 4.6C), but no significant differences in shoot production were observed after 21 d tissue culture regardless of H_2O_2 concentrations tested (Fig. 4.6C). Treating axes with 0.1 – 10 mM GSH had no effect on viability or growth (not shown), and was therefore not used as a treatment for axes isolated from seeds desiccated beyond 5 d. Treating embryonic axes isolated from 10 d or 21 d desiccated seeds with 0.1 mM H_2O_2 did not significantly ($P < 0.05$) affect viability (not shown) or vigour (Fig. 4.6D).

The effect on the percentage of axes infected by soaking embryonic axis in various concentrations of H_2O_2 is shown in Figure 4.6E. Increasing the concentration of H_2O_2 from 0 to 10 mM decreased the incidence of infection.

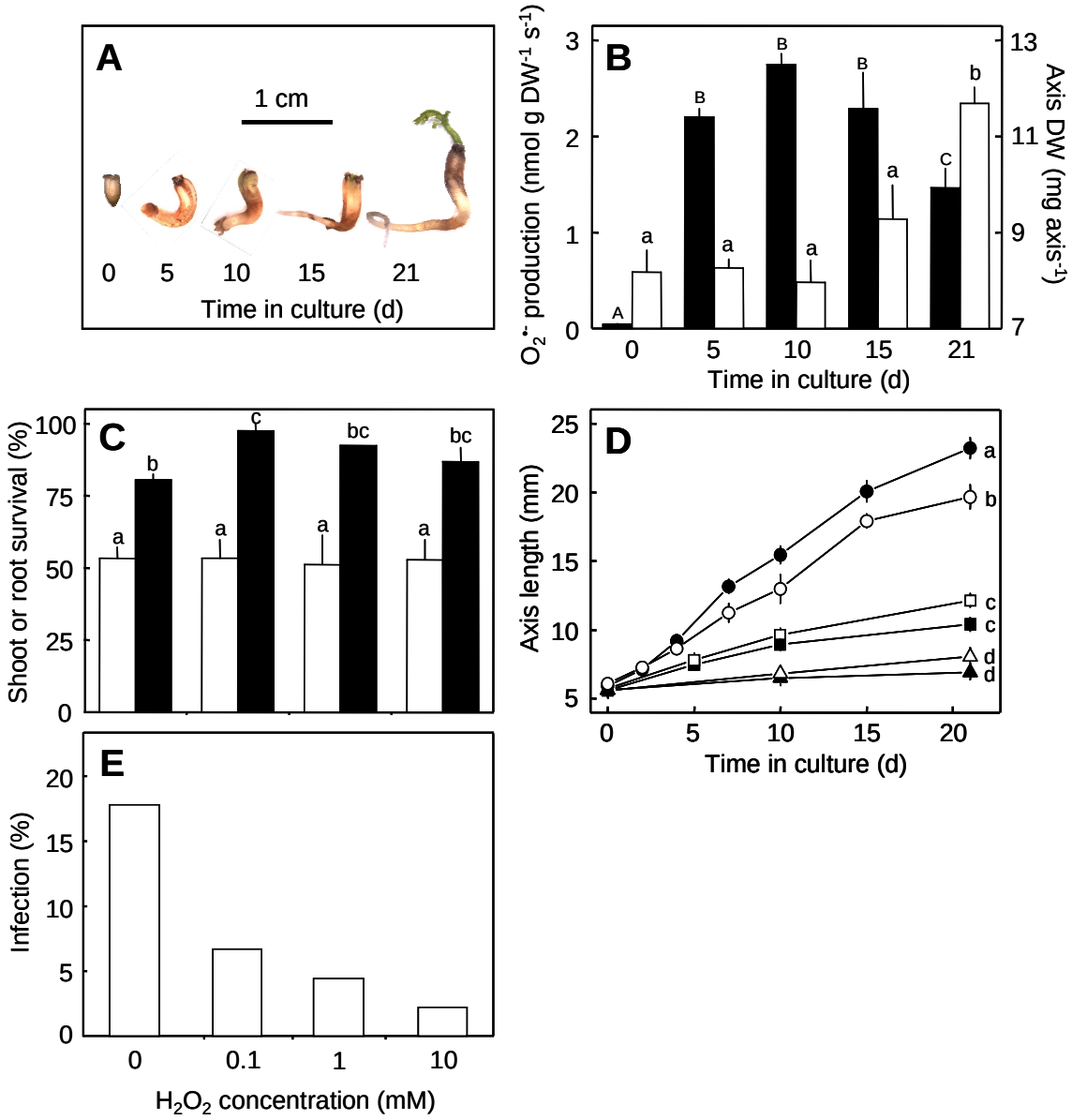


Figure 4.6 Superoxide production during growth of isolated *Castanea sativa* embryonic axes from seeds desiccated for 5 d and the effect of an H_2O_2 application on growth. **[A]** Snapshots of a typical embryonic axis. **[B]** Extracellular $O_2^{\bullet-}$ production by embryonic axes (left y-axis, black bars) during growth, expressed as increase in DW (right y-axis, white bars) (means ± SE, n = 4 replicates of 5 axes). **[C]** Effects of various concentrations of H_2O_2 on the survival of roots (black bars) and shoots (white bars), expressed as the percentage of embryonic axes that displayed root or shoot elongation by more than 5 mm. Embryonic axes were treated for 1 h immediately after excision. **[D]** Effect of H_2O_2 on the vigour of embryonic axes, expressed as axis elongation, excised from seeds desiccated at 15 % RH for 5 d (circles), 10 d (squares) and 21 d (triangles). Open and closed symbols denote axes that were soaked in H_2O and 0.1 mM H_2O_2, respectively, for 1 h (means ± SE, n = 3 replicates of 15 axes each, different letters denote significant (P < 0.05) difference after 21 d culturing). **[E]** Effect of various concentration of H_2O_2 on the percentage of axes that became infected during 21 d culturing.

4.3 Discussion

In chapter 3, it was shown that extracellular $O_2^{\bullet-}$ production increased with desiccation before major viability loss suggestive of a role in stress response and hypothesised to be a stress signal. Here, ECPOX were identified to be capable of $O_2^{\bullet-}$ production, with a putative role for phenolic acids as apoplastic reductants. Moreover, evidence is provided that extracellular ROS production has a positive role in facilitating survival of desiccation stressed seeds and embryonic axes.

4.3.1 Reactive oxygen species production at the surface of *Castanea sativa* embryonic axes

The staining of $O_2^{\bullet-}$ and H_2O_2 in the presence of ECPOX at the embryonic axis cut edge (Fig. 4.2) shows that ROS-producing enzymes are concentrated at the surface of the axis. Here, the axis will be most capable to sense its environment and will also be the primary site of any pathogen challenges. It is therefore a location expected to show heightened signalling and defence mechanisms that could involve ROS, in agreement with the intensity of staining observed at the embryonic axis surface. The image also shows ROS production in the area of the embryonic axis where the vascular bundle lies, which in mature plants is heavily lignified. ECPOX are essentially involved in the oxidative coupling of phenolic acids during lignification (Wallace and Fry, 1999; Ros-Barceló *et al*., 2004) and are likely to be found in vascular bundles. Lignin pre-cursors, such as ferulic acid, could be concentrated here so that the tissue is prepared for growth (Tokunaga *et al*., 2009). Therefore, ROS could have been produced by ECPOX using phenolic acids as reductants in response to wounding.

Plants require the rapid healing of wounded tissue to avoid pathogens from gaining entrance. In chapter 3, the magnitude of the oxidative burst following embryonic axis excision was estimated to be sufficiently powerful to sterilise the wounded site. However, ROS can serve other physiological roles, such as reinforcing damaged or infected cell walls by rapid H_2O_2-dependent

oxidative cross-linking of existing cell wall proteins (Bradley *et al.*, 1992; Otte and Barz, 1996). An accumulation of phenolic compounds (Pearce *et al.*, 1998; Hagel and Facchini, 2005), localised increases in ECPOX transcription (Cook *et al.*, 1995) and ECPOX activity (Sasaki *et al.*, 2002; Ruuhola and Yang, 2006) have all been observed at wounding sites. ECPOX have been suggested to be involved in $O_2^{\bullet -}$ production (Kawano, 2003; Minibayeva *et al.*, 2009). However, the observation of ECPOX activity and ROS production at the same location, such as wounding sites, only indicates that ECPOX are involved in ROS metabolism and not that they are responsible for their production. Hence, more work was carried out to gain evidence for ECPOX-mediated $O_2^{\bullet -}$ production.

4.3.2 A putative role for extracellular peroxidases in superoxide production

Measuring the wounding-induced production of $O_2^{\bullet -}$ by *C. sativa* embryonic axes in the presence of enzyme inhibitors provided evidence that ECPOX were partially responsible. The oxidative burst was sensitive to both NaN_3 and DPI, suggesting that enzymes producing apoplastic ROS include both ECPOX and NAD(P)H oxidase (Table 4.1). However, caution needs to be exercised when using enzyme inhibitors. Some reports indicate that DPI has low specificity as an inhibitor and does not only inhibit flavin-containing enzymes, such as NAD(P)H oxidase (Henderson and Chappell, 1996). However, DPI has also been reported to inhibit the $O_2^{\bullet -}$-generating step of ECPOX (Frahry and Schopfer, 1998), while other authors (Ros-Barceló and Ferrer, 1999) found that DPI does not affect the $O_2^{\bullet -}$-generating step of ECPOX via compound III (for ECPOX pathways of ROS metabolism and production see Fig. 4.1). Sodium azide inhibits hemoproteins such as ECPOX (Liu *et al.*, 2006) rather than flavin-containing NAD(P)H oxidase. Hence, the sensitivity of $O_2^{\bullet -}$ production to NaN_3 suggests that ECPOX are involved in $O_2^{\bullet -}$ production in *C. sativa* axes, although ROS production by other ROS producing enzymes can not be discounted.

ECPOX activity could readily be detected in cell wall fractions (Fig. 4.3A). On a DW basis, much higher ECPOX activities were found in embryonic axes compared to the cotyledons suggesting that they play a greater role in the metabolism of the axis. Within embryonic axes, most ECPOX activity was found in the crude cell extract dominated by intracellular components, but considerable activities were also found in the cell wall fraction containing ionically bound proteins (B3). In the presence of NADH, the B3 fraction of the axis could also be stimulated to produce more $O_2^{\bullet-}$ than any other (Fig. 4.3B), including the crude cell fraction that is likely to contain NAD(P)H oxidase. As non-specific spectrophotometric measurements in the presence of SOD were not taken, the epinephrine assay can only be used for an indication of $O_2^{\bullet-}$ production by cell wall fractions. We could expect the specificity of the assay to be *c.* 60 % (Table 4.1), and therefore the actual amount of $O_2^{\bullet-}$ produced by ionically bound proteins in the presence of 0.1 mM NADH and ferulic acid is estimated to be 15 and 100 nmol g^{-1} DW s^{-1}, respectively. H_2O_2 was essential for $O_2^{\bullet-}$ production (Fig. 4.3C), suggesting H_2O_2 concentrations could influence the assay, although this was not tested here. Without reductants some $O_2^{\bullet-}$ was produced in support of compound III being involved with $O_2^{\bullet-}$ production (Fig. 4.3C; Fig. 4.3D). H_2O_2 can be provided by several extracellular enzymes, such as AOX (Allan and Fluhr, 1997; Cona *et al.*, 2006), or via $O_2^{\bullet-}$ disumtation. The presence of either NADH or phenolic acids increased rates of $O_2^{\bullet-}$ production (Fig. 4.3), suggesting that the major pathway responsible for producing $O_2^{\bullet-}$ was via the conventional peroxidase cycle and not via compound III (Fig. 4.1). Therefore, DPI is more likely to inhibit ECPOX-mediated $O_2^{\bullet-}$ production.

The correlations of high ECPOX activity and $O_2^{\bullet-}$ production in the presence of ferulic acid in the B3 fraction suggests that ionically bound cell wall ECPOX could be involved in producing $O_2^{\bullet-}$. According to Fig. 4.1, ferulic acid could be oxidised to a phenoxyl radical, reducing $^3O_2$ to $O_2^{\bullet-}$ (Takahama, 2004). Unbound ferulic acid has been identified in the apoplast of maize (Šukalović *et al.*, 2005) and pea roots (kukavica *et al.*, 2009). The in-gel

activity stain revealed a 63 kDa ionically-bound protein that was capable of producing $O_2^{\bullet-}$ using ferulic acid as a reductant, but only in the presence of H_2O_2 suggestive of an ECPOX (Fig 4.4). Staining in the presence of NADH, although stronger than with ferulic acid, was H_2O_2 insensitive suggesting some non-specific activity. The enzyme size of 63 kDa is large for the MW of a typical ECPOX, usually between 28 to 60 kDa (Hiraga *et al.*, 2001), and as no staining occurred in the presence of the classic ECPOX substrate guaiacol, the identity of the extracellular $O_2^{\bullet-}$-producing enzyme remains to be confirmed by further enzyme purification and sequencing. However, all other data indicate that ECPOX are involved in producing $O_2^{\bullet-}$ in response to abiotic stress.

4.3.3 Hydrogen peroxide prevented desiccation-induced losses during seed germination and enhanced embryonic axis growth

There are several possible, non-exclusive explanations for the enhanced total germination after H_2O_2 treatment of desiccated seeds (Fig. 4.5C) and embryonic axes (Fig. 4.6C; Fig. 4.6D). The prevalence of infection on un-treated and non-desiccated seeds can be seen in Figure 3.3A. H_2O_2 could have directly surface sterilised the seeds as less infections were observed in desiccated seeds treated with H_2O_2 (data not shown). The greatest improvements in germination occurred in seeds with decreased vigour that germinated later than the non-desiccated controls, with less vigorous seeds providing more time for pathogens to outcompete seed defence mechanisms. Indeed, ROS are directly toxic to invading pathogens (Murphy *et al.*, 1998), and are produced immediately following wounding (Fig. 3.8) where an opportunity is provided for pathogens to gain entry. However, downstream of the immediate impacts of ROS, defence related genes inducible by wounding, have shown to be up-regulated by exogenous applications of H_2O_2 (Le Deunff *et al.*, 2004) or down-regulated by inhibiting its production (Orozco-Cardenas *et al.*, 2001). Embryonic axes were surface sterilised in 50 mM sodium dichloroisocyanurate before growth, but a clear trend was observable of fewer

cases of infection when higher concentrations of H_2O_2 were used (Fig. 4.5E). Axes that had perished from desiccation generally became infected, but growth of axes that had survived desiccation occurred rapidly, suggesting that infection was a post-mortem effect. However, fewer viable axes became infected during growth following treatment with H_2O_2, indicating that H_2O_2 had initiated defence responses and that sterilisation alone may be too simplified an explanation.

It will be interesting to see whether different genes related to defence are expressed in response to H_2O_2 treatments. ROS have also been implicated in the control of cell division for development, growth and differentiation (Foreman *et* al., 2003; Liszkay *et al*., 2004; Gapper and Dolan, 2006) and could also be involved in the removal of unwanted cells by initiating programmed cell death (Raff, 1998; Laloi *et al*., 2004; Gechev *et al*., 2006). Interestingly, mild desiccation has been occasionally reported to stimulate total germination in some recalcitrant seeds, including *Acer pseudoplatanus* (Daws *et al*., 2006), *Aesculus hippocastanum* (Tompsett and Pritchard, 1998), *Ekebergi capensis* (Pammenter *et al*., 1998), *Litchi chinensis* (Fu *et al*., 1994), and *Quercus robur* (Kranner *et al*., 2006). Germination stimulated by mild desiccation has been attributed to a continuation of maturation (Tompsett and Pritchard, 1998; Daws *et al*., 2006), but desiccation-induced production of ROS may also contribute. Here, it was shown that mild desiccation induced $O_2^{\bullet -}$ production (Fig. 3.9) and that H_2O_2 could promote germination (Fig. 4.5C).

Although H_2O_2 was applied exogenously at high concentrations to whole seeds, these concentrations would have decreased considerably as H_2O_2 diffused through the seed coat and cotyledons to the axes. High H_2O_2 concentrations can be rapidly broken down by catalases and class III peroxidases working in a catalase like function (Hernández-Ruíz *et al*., 2001), in agreement with the bubbling shown in Figure 4.5A. Furthermore, exogenous applications of H_2O_2 could stimulate endogenous $O_2^{\bullet -}$ production by peroxidases (Halliwell, 1978; Hiner *et al*., 2001).

4.3.4 Summary

In summary, results presented in chapter 4 show that H_2O_2 can prevent viability losses during germination of desiccated seeds and enhance growth of embryonic axes. Taken together with the observation that seeds and embryonic axes desiccated to (sub)lethal WCs may have lost the capabilities to induce $O_2^{\bullet-}$ production, the data suggests that extracellular production of ROS such as $O_2^{\bullet-}$ and H_2O_2 by embryonic axes in intact seeds may ameliorate the effects of stress. A role for extracellular phenolic acids, particularly ferulic acid, known to be available in the apoplast is proposed for ECPOX-mediated $O_2^{\bullet-}$ production. Evidence is provided that ROS are important to growth and development, and to the best of my knowledge the results presented here are the first to show that H_2O_2 can improve total germination of stressed recalcitrant seeds. Irrespective of the mechanism of action, treatment with H_2O_2 may be a useful tool for improving the viability of recalcitrant seeds and facilitating embryonic axis recovery from non-germinating seeds.

Chapter 5 Production of reactive oxygen species by *Pisum sativum* during seed germination and seedling desiccation

5.1 Introduction

Extracellular ROS play key roles in plant development (Foreman *et al*., 2003; Gapper and Dolan, 2006; Jones *et al*., 2007), but their significance for seed germination is poorly understood. Previously, many studies on ROS production in seeds have focused on their detrimental effects related to oxidative stress. For example, it has been reported that during the imbibition of *Glycine max* L. Merr. embryonic axes (Boveris *et al*., 1980; Boveris, 1984) and germination of *P. sativum* seeds (Wojtyla *et al.*, 2006) free radicals and ROS are generated. However, other studies have shown that ROS can regulate dormancy status (Fontaine *et al*., 1994; Naredo *et al*., 1998; Ogawa and Iwabuchi, 2001; Sarth *et al*., 2007; Oracz *et al*., 2009). Furthermore, some authors have suggested that ROS production within the endosperm promotes radicle protrusion by weakening the structures that restrict axis growth (Müller *et al*., 2006; 2009). Elevated rates of ROS have also been postulated to sterilise the emerging radicle from pathogens (Schopfer *et* al., 2001; Morkunas *et al*., 2004), in agreement with the participation of ROS in pathogen defence (Baker and Orlandi, 1995; Bolwell *et al*., 2002). Hence, ROS contribute to important processes during and shortly after seed germination.

Seed germination is usually associated with radicle rupture of the seed coat (Bewley and Black, 1994), which occurs via the expansion of embryonic cells (da Silva *et al*., 2008). Cells without secondary cell walls can expand if breakages are created within the existing primary cell wall structure. Cell-wall "loosening" (Schopfer, 2001) is, in part, mediated by hydrolytic enzymes (Finch-Savage and Leubner-Metzger, 2006). However, ECPOX are capable of loosening cell-walls by the production of $^{\bullet}OH$, which cleaves cell wall

polysaccharide chains allowing cellular expansion (Chen and Schopfer, 1999; Schopfer, 2001; Liszkay *et al*., 2004; Kukavica *et al*., 2009). An influence of ECPOX on root growth was shown by Passardi *et al*. (2006), who demonstrated that up-regulating or partially blocking the activity of two particular ECPOX in Arabidopsis enhanced or retarded growth, respectively. Contrary to promoting elongation, ECPOX activity can restrict cellular expansion through lignification by coupling cell wall lignols in the presence of H_2O_2 (Fry, 1986; Ros-Barceló *et al*., 2004). Furthermore, ECPOX can be induced to produce ROS in response to many different stresses (Castillo *et al*., 1984; Markkola *et al*., 1990; Bolwell *et al*., 1995; McLusky *et al*., 1999). Thus, the diversity of ECPOX activities not only contributes to the roles of ROS in seedlings during normal growth, but also is also essential in response to a multitude of stresses.

Desiccation tolerance is lost near or after radicle protrusion (Dasgupta *et* al, 1982; Seneratna and Mckersie, 1983; Reisdorph and Koster, 1999). However, in a limited time window after germination, the desiccation tolerance of some seedlings can be induced back by osmotic priming. This has been used to better understand desiccation tolerance mechanisms (Buitink *et al*., 2003; Leprince *et al*., 2004; Faria *et al*., 2005). Conversely, the transition from desiccation insensitive to sensitive stages of orthodox seeds has been used to create a model for studying seed recalcitrance (Sun, 1999). The radicle meristem of seedlings is particularly sensitive to even slight desiccation, but mildly desiccation stressed seedlings can survive by growing new adventitious roots (Farrant *et al*., 2004). In chapter 3, it was shown that recalcitrant *C. sativa* embryonic axes produced a Gaussian-type response of extracellular $O_2^{\bullet-}$ associated with desiccation. During the early stages of water loss elevated rates of $O_2^{\bullet-}$ production was observed. Maximum production of $O_2^{\bullet-}$ occurred before major viability loss, indicating that $O_2^{\bullet-}$ could have been a signalling response to desiccation stress rather than a consequence of unbalanced metabolism and necrosis. Furthermore, treating mildly desiccation stressed *C. sativa* seeds and embryonic axes with H_2O_2

improved viability and growth rates, revealing that pro-oxidants can help mitigate desiccation stress.

The initial aims of this chapter were to investigate the patterns and mechanisms of extracellular $O_2^{\bullet-}$ and H_2O_2 production during germination and early seedling development of *P. sativum*. As mentioned previously, seed ageing is associated with intracellular oxidative stress (Wojtyla *et al*., 2006), but the effects of seed ageing on the production of extracellular ROS is unknown. Here, the production of extracellular $O_2^{\bullet-}$ production during the germination of aged and non-aged seeds was measured. Conductivity measurements of electrolyte leakage in leachates from aged and non-aged seeds were also taken to provide an indication of membrane damage, a typical marker of stress (Sattler *et al*., 2004). The enzymes that contribute to ROS production during germination were investigated using several assays; first, by measuring ROS production of seedlings in the presence of enzyme inhibitors, second, by separating extracellular ROS-producing enzymes using a cellular fractionation and third, using PAGE in conjunction with in-gel staining for identifying extracellular enzymes capable of $O_2^{\bullet-}$ production.

In the second part of this chapter, the responses of seed and seedling axes of desiccation tolerant and intolerant *P. sativum* seeds and seedlings, respectively, were investigated. Hypothesising that seedling axes (desiccation intolerant) will behave similar to recalcitrant *C. sativa* embryonic axes in their desiccation response, but embryonic axes from non-germinated seeds (desiccation tolerant) will not, the effect of desiccation on the production of $O_2^{\bullet-}$ following various periods of desiccation was measured. In *P. sativum* seedling axes, mild desiccation was sufficient to kill the radicle meristem, but seedling viability was retained with new adventitious root growth. The participation of $O_2^{\bullet-}$ production within the survival zone was further characterised and the effects of an exogenous application of H_2O_2 on the survival of *P. sativum* seedlings following mild desiccation was tested.

5.2 Results

5.2.1 Effects of seed ageing on viability, membrane damage and extracellular $O_2^{\bullet-}$ production

In non-aged seeds, first radicle emergence occurred *c.* 20 h after imbibition was initiated (Fig. 5.1A). Ageing *P. sativum* seeds at 45 ^{o}C and 72 % RH (WC = 11.7 %) decreased their ability to germinate; total germination (TG) was reduced from 98.0 ± 1.2 % in non-aged seeds to 77.0 ± 4.4 % and 0 % in seeds aged for 23 (data not shown) and 102 d (Fig 5.1A), respectively. Seeds aged for 23 d also took longer to germinate with first radicle emergence occurring 45 h after imbibition was initiated. Conductivity measurements of seed leachates, indicating changes in membrane permeability were also taken as a separate viability indicator. Significant differences ($P < 0.05$) in conductivity occurred after 3.2 h of soaking, when dead seeds began to leak significantly more electrolytes than non-aged seeds. A significant difference ($P < 0.05$) between non-aged and 23 d aged seeds was found after 4.0 h of soaking. After 12 h of imbibition, seeds aged for 23 d and 102 d, with corresponding viabilities of 77 % and 0 %, respectively, leaked 2.4 and 3.4 times more electrolytes, respectively, than non-aged seed (Fig. 5.1B).

Aged and non-aged seeds were then measured for their ability to produce ROS during germination. The epinephrine assay for measuring $O_2^{\bullet-}$ suggested an immediate production of ROS, from now referred to as the initial burst, which accompanied seed imbibition (Fig. 5.1C). Aged seeds (77 % and 0 % TG) displayed only half the initial burst compared to non-aged seeds. The initial burst was a one-off event; desiccating seeds after 1 h imbibition back to their original WC and re-measuring ROS production revealed no further production (data not shown). Interestingly, epinephrine measurements of the initial burst were insensitive to SOD and hence, not a consequence of $O_2^{\bullet-}$ production (Table 5.1). Furthermore, neither ECPOX nor NAD(P)H oxidase inhibitors had any significant ($P < 0.05$) effect upon

adrenochrome formation. To measure the production of $O_2^{\bullet -}$ during germination, seeds were incubated in epinephrine at regular intervals for 30 min and placed back on germination paper between measurements. In non-aged seeds, a steady increase in adrenochrome was observed at *c.* 30 h when around half of the seeds had germinated (Fig. 5.1A; Fig. 5.1C). In comparison, the formation of adrenochrome in the incubation solution by seeds aged for 23 d occurred later and at a lower rate. Seeds aged for 102 d never germinated and produced no adrenochrome beyond the initial burst. For non-aged seeds, no significant ($P < 0.05$) differences were observed in TG regardless of whether measurements of $O_2^{\bullet -}$ production were made. However, TG during $O_2^{\bullet -}$ production measurements for seeds aged for 23 d was 33 % lower, showing that repeated incubation in epinephrine was stressful.

Table 5.1 Effect of enzyme inhibitors on the initial oxidative burst when *Pisum sativum* seeds were first imbibed. Superoxide production was measured by incubating seeds in 5 ml of 1 mM epinephrine for 30 min (control) and the specificity of the assay was tested by adding 200 units ml^{-1} of SOD. Measurements were also taken in the presence of the ECPOX inhibitor NaN_3 and the NAD(P)H oxidase inhibitor DPI (means ± SE, n = 3 replicates of 10 seeds). There were no significant differences between treatments found ($P < 0.05$).

	Adrenochrome formation (pmol g DW^{-1} s^{-1})
Control	73.6 ± 8.3 (100 %)
+ SOD	71.9 ± 5.1 (98 %)
+ NaN_3 (1 mM)	77.8 ± 13.6 (106 %)
+ DPI (10 µM)	74.7 ± 13.9 (101 %)

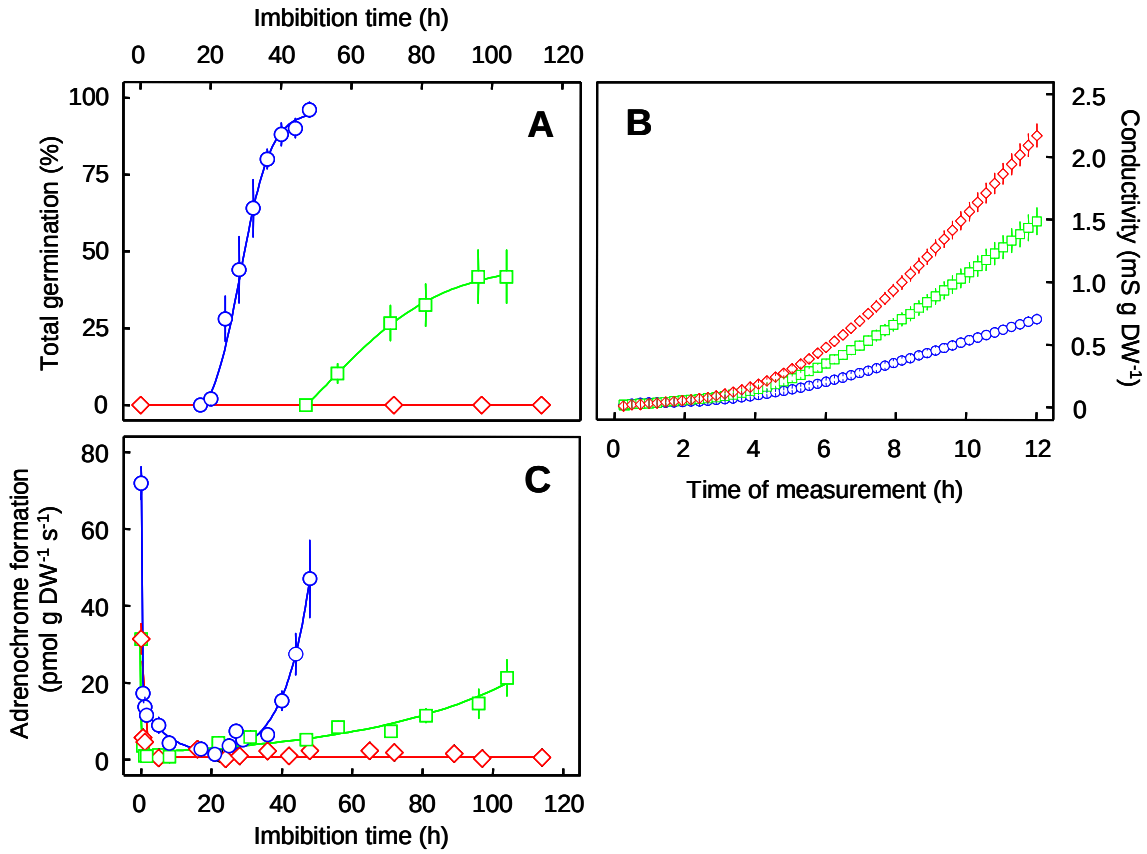


Figure 5.1 Total germination, membrane permeability and extracellular $O_2^{\bullet -}$ production by artificially aged *Pisum sativum* seeds. In [A, B and C] blue circles denote non-aged seeds. Green squares and red rhombs denote seeds that had been aged for 23 d and 102 d, respectively, at 72 % RH and 45 ^{O}C (WC = 11.7 %). **[A]** Germination of seeds used in measurements of $O_2^{\bullet -}$ production is shown and no further germination occurred after the last data point. **[B]** Conductivity measurements were made of leachates produced by soaking individual desiccated seeds in 3.0 ml of H_2O for up to 12 h (mean ± SE, n = 28). **[C]** Rates of extracellular $O_2^{\bullet -}$ production, as assessed by adrenochrome formation, were made by incubating 10 seeds for 30 min in 5 ml of 1 mM epinephrine. Measurements were taken until TG, as shown in [A], had been reached. The same seeds were used for the whole time course (mean ± SE, n = 5 replicates of 10 seeds).

5.2.2 The production of extracellular $O_2^{\bullet-}$ and H_2O_2 during germination

To identify which tissue produces $O_2^{\bullet-}$ during germination, separate measurements of the embryo and seed coat were made (Fig. 5.2A). The embryo was responsible for the vast majority of $O_2^{\bullet-}$ production, with negligible contribution by the seed coat. In Figure 5.2A, the data is expressed per seed, allowing relative comparison of $O_2^{\bullet-}$ production by the seed coat and embryo, whereas in all other figures $O_2^{\bullet-}$ production is expressed on a DW basis. Staining the embryo with NBT revealed that only the seedling axis produced $O_2^{\bullet-}$ (not shown). Staining seedling axes with NBT revealed that an area of a few millimetres behind the root meristem was particularly intensive in producing $O_2^{\bullet-}$ (Fig. 5.2B). Patchy staining also occurred across the whole surface and increased with radicle elongation. As extracellular H_2O_2 could not be detected during germination (Fig. 5.2C), only $O_2^{\bullet-}$ production was further characterised. Measurements 48 h after the onset of imbibition showed that adrenochrome was produced at a rate of 33 - 35 pmol g DW^{-1} s^{-1} (Table 5.2). Assuming that the epinephrine assay is considered at this point to *c.* 75 % specific to $O_2^{\bullet-}$ (Fig. 5.2D), this indicates an $O_2^{\bullet-}$ production rate of 25 pmol g DW^{-1} s^{-1}. Seedlings were separated by radicle size after TG had been reached, allowing a comparison of $O_2^{\bullet-}$ production in seedlings at different developmental stages. Using this approach, Figure 5.2D shows that $O_2^{\bullet-}$ production only occurs when the radicle length was longer than 10 mm, in agreement with the delay of $O_2^{\bullet-}$ production after germination (Fig. 5.1A; Fig. 5.1C). Inhibiting ECPOX or NAD(P)H oxidase in seedlings imbibed for 48 h led to a decrease in adrenochrome formation by *c.* 40 %, suggesting that both enzymes contribute to $O_2^{\bullet-}$ production (Table 5.2).

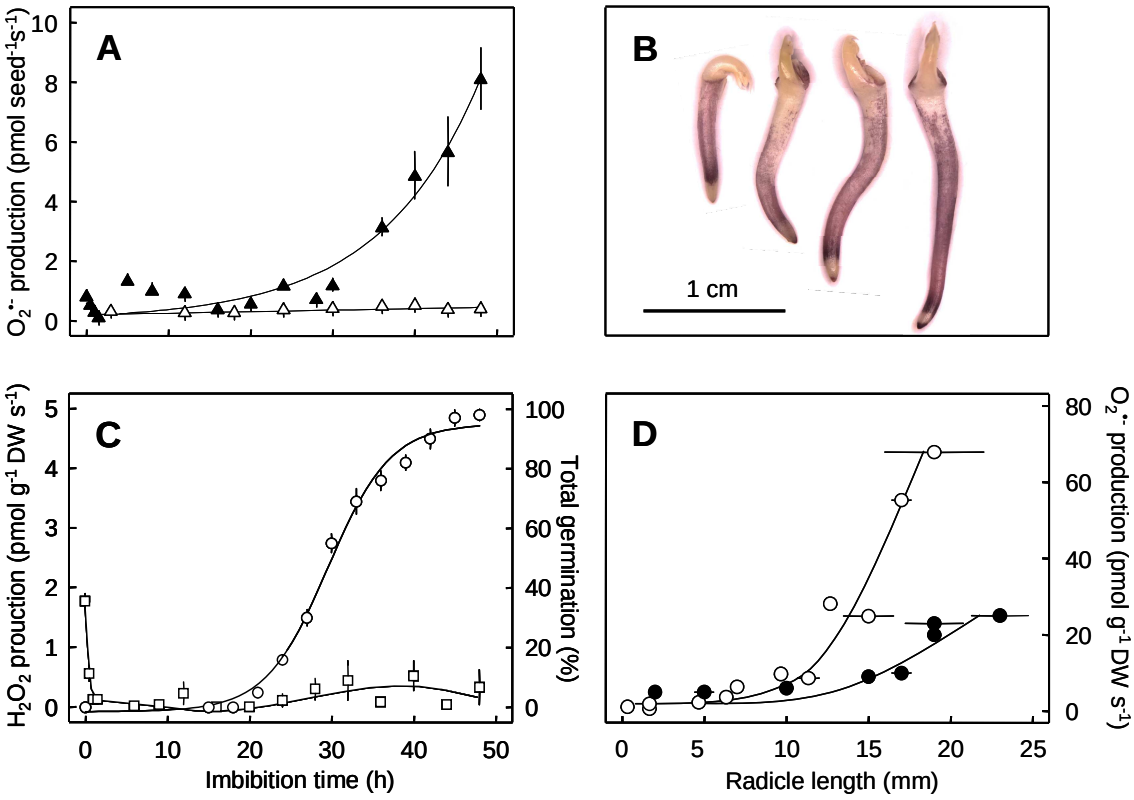


Figure 5.2 Production of extracellular $O_2^{\bullet-}$ and H_2O_2 during germination and seedling development of *Pisum sativum*. $O_2^{\bullet-}$ production is shown [A; C] during the first 48 h of imbibition or [B; D] in seedlings of various radicle lengths 56 h after imbibition was initiated or. **[A]** Contribution of embryos (closed triangles) and seed coats (open triangles) to extracellular $O_2^{\bullet-}$ production shown on a per-seed basis. Seed coats were separated from embryos after 3 h of soaking. Both were then fully re-desiccated before measurements. Embryos were imbibed at 25 °C on damp germination paper and $O_2^{\bullet-}$ production was measured using the same material throughout the time course. Different seed coats were used for $O_2^{\bullet-}$ measurements at each interval, which were kept hydrated from the beginning of the time course until reaching the respective time interval (n = 5 replicates of 10 embryos or seed coats each). **[B]** $O_2^{\bullet-}$ production, indicated by purple staining, was visualised by incubation seedling axes in 5 mM NBT for 5 min. The rate of $O_2^{\bullet-}$ production was quantified by incubating seeds, embryos or seed coats in 5 ml of 1 mM epinephrine for 30 min. **[C]** Total germination (circles) is shown at 25 °C, as used in all experiments (mean ± SE, n = 5 replicates of 20 seeds). H_2O_2 production (squares) during germination was quantified using the xylenol orange assay, courtesy of Miss Mariyana Ivanova, by incubating material for 30 min in distilled water and measuring the amount of H_2O_2 in 0.5 ml of the leachate (mean ± SE, n = 5 replicates of 10 seeds). **[D]** $O_2^{\bullet-}$ production dependent on radicle length. Measurements in the presence or absence of superoxide dismutase are represented by closed and open symbols, respectively (mean± SE of radicle length, n = 3 seedlings / measurement).

Table 5.2 Effect of enzyme inhibitors on $O_2^{\bullet-}$ production by *Pisum sativum* seedlings. $O_2^{\bullet-}$ production was measured by incubating seedlings after 48 h of imbibition in 5 ml of 1 mM epinephrine for 30 minutes (Control). Measurements were also taken in the presence of the ECPOX inhibitor NaN_3 or the NAD(P)H oxidase inhibitor DPI (means ± SE, n = 4 replicates of 6 seedlings).

	Adrenochrome formation (pmol g DW^{-1} s^{-1})
Control	35.3 ± 2.7 (100 %) ^{A}
+ NaN_3 (1 mM)	21.9 ± 1.3 (62 %) ^{B}
+ DPI (10 µM)	21.4 ± 2.1 (61 %) ^{B}

5.2.3 A role for ECPOX in extracellular $O_2^{\bullet-}$ production

ECPOX activity occurred mostly in the radicles; very little was detected in the cotyledons and seed coats (Table 5.3). Isolating and separating extracellular proteins by electrophoresis from non-germinated seeds and then staining gels with guaiacol and TMB, typical ECPOX substrates, produced a weak band indicative of a *c.* 200 kD ECPOX (Fig. 5.3A). During seedling development, as indicated by radicle length in Figure 5.3A, new ECPOX isoforms of smaller molecular mass were synthesised, but unfortunately the resolution of the bands in the gel was not clear and specific sizes were not revealed. ECPOX activity in the leachates increased almost exponentially during seedling development (Fig. 5.3B), as quantified using the ECPOX-catalysed H_2O_2-dependent oxidation of ABTS. Staining for enzyme activity after PAGE in the presence of 0.5 mM NADH and NBT indicated that non-germinated seeds did not contain NBT-reducing ($O_2^{\bullet-}$-producing) extracellular enzymes (Fig. 5.3C). During seedling development, extracellular enzymes of molecular masses of *c.* 200, 70 and 45 kD capable of producing $O_2^{\bullet-}$ were synthesised, with an additional 90 kD enzyme found in the crude cell fraction (Fig. 5.3C). To further investigate the activity of ECPOX, various reductants at different concentrations were tested for their ability to act as reductants for ECPOX-

induced in-gel $O_2^{\bullet-}$ production. Although NADH (Fig. 5.3C) and NADPH (not shown) could induce $O_2^{\bullet-}$ production in other proteins, only the *c.* 200 kD ECPOX (Fig. 5.3A) could be stimulated to produce $O_2^{\bullet-}$ using H_2O_2 in the presence cysteine (0.01 – 0.5 mM) or ferulic acid (only at 0.05 mM) (Fig. 5.3D).

Table 5.3 Activity of ECPOX in cell fractions of seedling axes, cotyledons and seed coats of *Pisum sativum* seedlings after 48 h imbibition. C, crude cell fraction; B1, proteins loosely associated to the cell wall; B2, proteins bound by hydrophobic interactions; B3, proteins bound by ionic bonds; B4, un-removable cell wall proteins (mean ± SE, n = 5 replicates of 25 seeds used for each fractionation, courtesy of Miss Mariyana Ivanova).

	POX activity (nkat g DW^{-1})		
Fraction	Seedling axis	Cotyledon	Seed coat
C	917 ± 63	24 ± 2	0.08 ± 0.00
B1	236 ± 7	8 ± 0	0.06 ± 0.00
B2	32 ± 1	1 ± 0	0.03 ± 0.00
B3	72 ± 1	5 ± 0	0.30 ± 0.01
B4	5 ± 0	0	0.04 ± 0.01

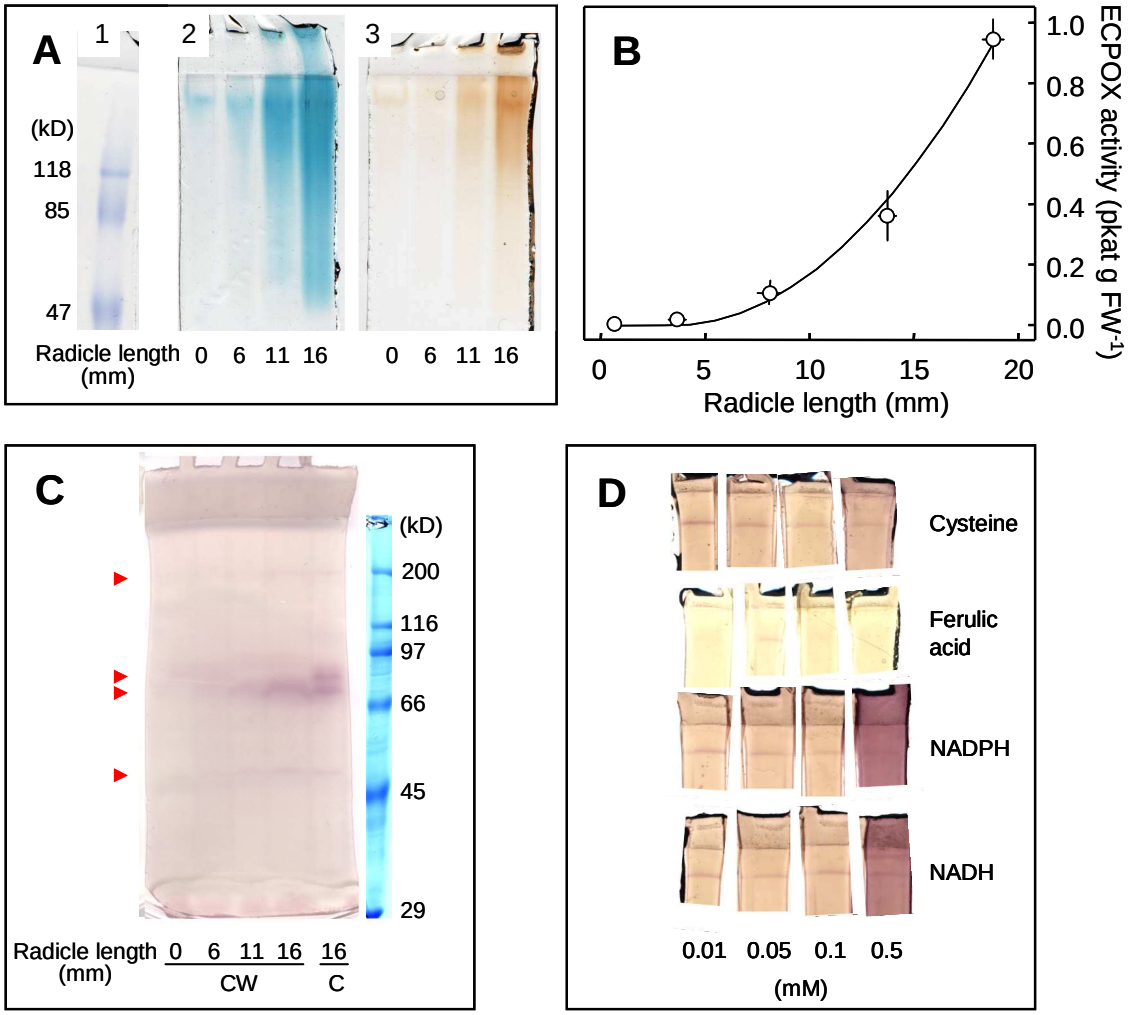


Figure 5.3 Characterisation of ECPOX and $O_2^{\bullet-}$-producing enzymes in axes of 48 h imbibed *Pisum sativum* seedlings. Extracellular enzymes (cell wall fractions B1 - B3) were isolated and then separated by electrophoresis before in-gel activity staining [as shown in A, C and D]. **[A]** "gel 1" shows the pre-stained marker, indicating molecular masses (kD). ECPOX activity was detected with TMB and guaiacol, as shown in "gel 2" and "gel 3", respectively, in seedlings with radicle lengths of 0 ± 0, 6 ± 1, 11 ± 1 and 16 ± 2 mm of gel lanes 1, 2, 3 and 4, respectively. **[B]** ECPOX activity in leachates of *P. sativum* seedlings of various radicle sizes, as measured by the H_2O_2-dependent oxidation of ABTS during 30 min incubation (mean ± SE, n = 5 replicates of 10 seedlings in 5 ml). **[C]** $O_2^{\bullet-}$ production by enzymes was visualised using NBT in the presence of 0.5 mM NADH without H_2O_2, using the same cell wall fractions (CW) used in [A] and the crude cell fraction (C) of seedlings with 16 ± 2 mm length radicles. Molecular mass marker (kD) was stained with Coomassie brilliant blue. **[D]** $O_2^{\bullet-}$-producing enzymes corresponding to *c.* 200 kD size in seedling axes with radicle lengths of 16 ± 2 mm, were visualised using NBT in the presence of various reductants (0.01 - 0.5 mM) and 10 mM H_2O_2.

5.2.4 The effect of imbibition time on desiccation tolerance of seeds and seedlings

Germination of *P. sativum* seeds was accompanied by a loss of desiccation tolerance, but in some seeds this occurred before radicle emergence. During seed imbibition, water uptake occurred rapidly; within 16 h of imbibition the embryonic axis WC increased from 6.6 ± 0.1 % to 71.1 ± 1.9 % (n = 10), but no germination was observed at this point (Fig. 5.4A). However, 20 % of seeds that had been desiccated after 16 h of imbibition were unviable (Fig. 5.4B). The axis WC at the point of radicle emergence, which peaked between 20 to 50 h (Fig. 5.1A; Fig 5.2C), was 83.9 ± 0.5 % (n = 16). The average axis length after 56 h imbibition was 9.0 ± 1.5 mm (Fig. 5.4A), but at this point some seeds had just germinated whereas others had radicle lengths greater than 20 mm and displaying root hair growth, reflecting the variability in vigour in the population. The interception point between the sigmoidally fitted curves of TG during imbibition and the viability after subsequent desiccation of the same seeds was below 50 % (Fig. 5.4B). It seems that some seeds had lost desiccation tolerance before germination, perhaps due the time spent at intermediate WCs during desiccation as discussed in chapter 3.

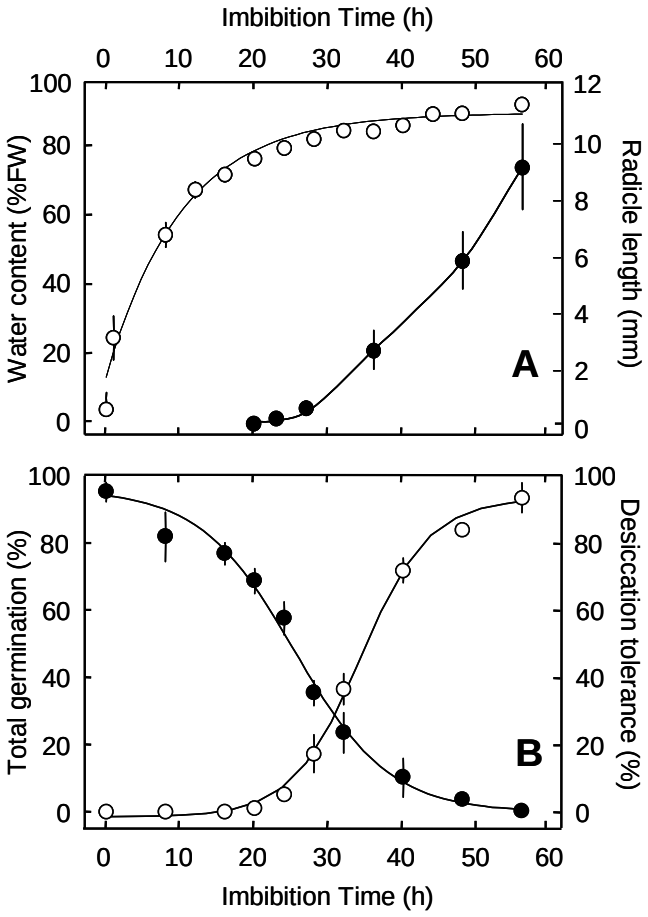


Figure 5.4 Loss of desiccation tolerance during germination of *Pisum sativum* seeds. For germination, seeds were soaked for 1 h in distilled H_2O and then imbibed at 25 ^{O}C on germination paper. During imbibition, measurements of **[A]** WC (open symbols; left y axis), radicle length (closed symbols; right y axis), **[B]** TG, as indicated by radicle growth by more than 2 mm (open symbols; left y axis) and loss of desiccation tolerance (closed symbols; right y axis) were made. To measure the degree of desiccation tolerance during imbibition the seeds used for TG were desiccated at 15 % RH back to their initial WC (6.6 ± 0.1 %) and then rehydrated until TG had been achieved. Mean ± SE, n = 20 seeds in [A] and n = 3 reps of 20 seeds in [B].

5.2.5 The production of $O_2^{\bullet -}$ in response to desiccation

Axes isolated from seeds after 6 h or 56 h of imbibition, referred to as embryonic axes and seedling axes, respectively, showed substantial differences in producing extracellular $O_2^{\bullet -}$ after desiccation. Seedling axes took 12 times longer to reach a WC below 20 % than embryonic axes (Fig. 5.5A; Fig. 5.5B). In seedling axes, hypocotyls desiccated slower than radicles. For example, after 6 h of desiccation the WCs of the hypocotyl and radicle were 67.4 ± 3.5 % and 55.5 ± 4.8 %, respectively (Fig. 5.5B). Initially, desiccating seedling axes led to an increase in the production of extracellular $O_2^{\bullet -}$, doubling after 6 h desiccation (Fig. 5.5C). After 16 h of desiccation, rates of $O_2^{\bullet -}$ production had peaked. In comparison to the stress response of seedling axes, desiccating embryonic axes did not result in any increase in $O_2^{\bullet -}$ production (Fig. 5.5D). However, excising embryonic axes led

to a wounding-induced oxidative burst (2.8 ± 0.5 nmol g^{-1} DW s^{-1} during 30 min, n = 3 replicates of 5 axes), indicating that axes at this developmental stage were still capable of producing $O_2^{\bullet-}$. The wounding response is a focus of the next chapter and not dwelled upon here.

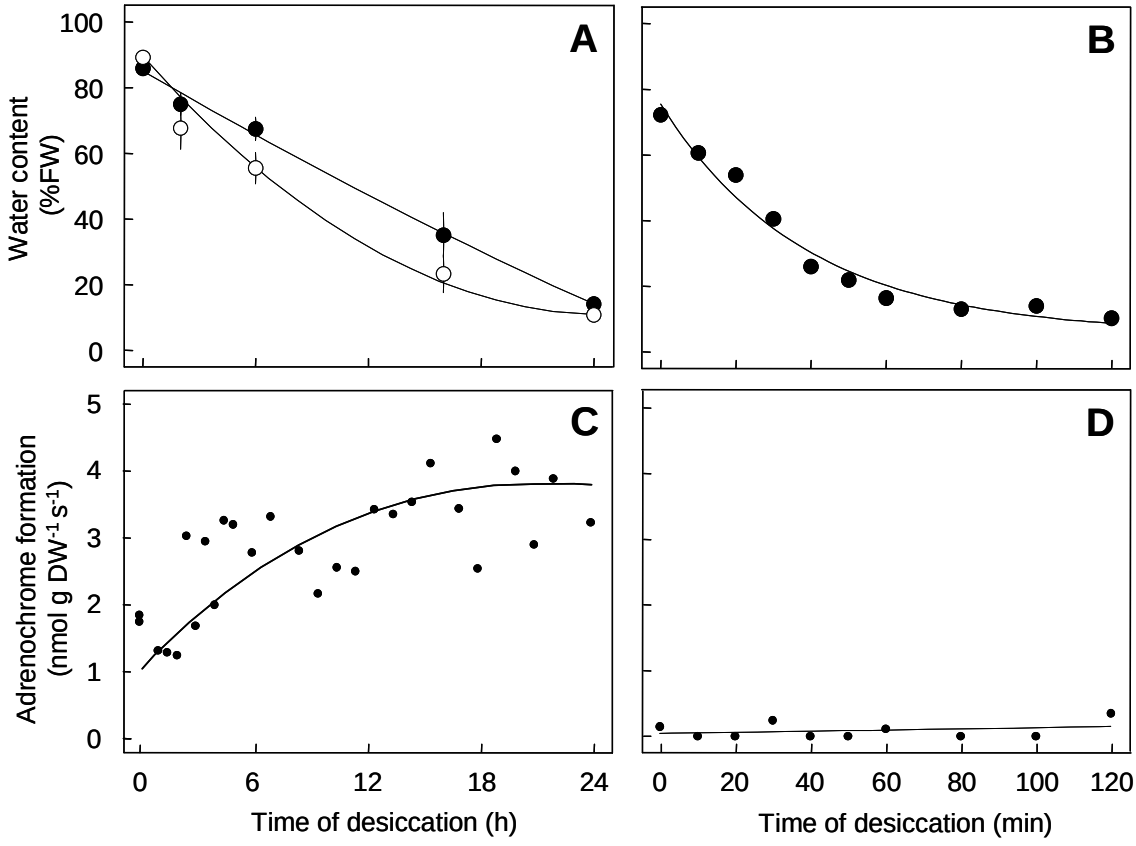


Figure 5.5 Effect of desiccation on WC and $O_2^{\bullet-}$ production by isolated *Pisum sativum* embryonic axes and seedling axes, isolated from 6 and 56 h imbibed seeds, respectively. For desiccation, axes were removed from seeds and placed over silica gel (3% RH) for various intervals. **[A]** WC of separated hypocotyls and radicles, as represented by closed and open symbols, respectively (mean ± SE, n = 10) of seedling axes. **[B]** WC of embryonic axes during desiccation (n = 5 axes per measurement). [C; D] Extracellular $O_2^{\bullet-}$ production, as measured by adrenochrome formation in 1.5 ml incubation solution of 1 mM epinephrine, by **[C]** Seedling axes or **[D]** embryonic axes following various periods of desiccation of (n = 5 axes per measurement).

It was noticed that in seedling axes epicotyls desiccated slower than radicles (Fig. 5.5A). Mapping the water loss across the tissue provided a more accurate picture of seedling axis desiccation, which started at the radicle tip and after 6 h migrated towards the epicotyl (Fig 5.6A). Between 6 h and 16 h of desiccation the epicotyl became fully desiccated, although other parts of the seedling retained a WC of up to 45 %. After 2 h of desiccation the radicle

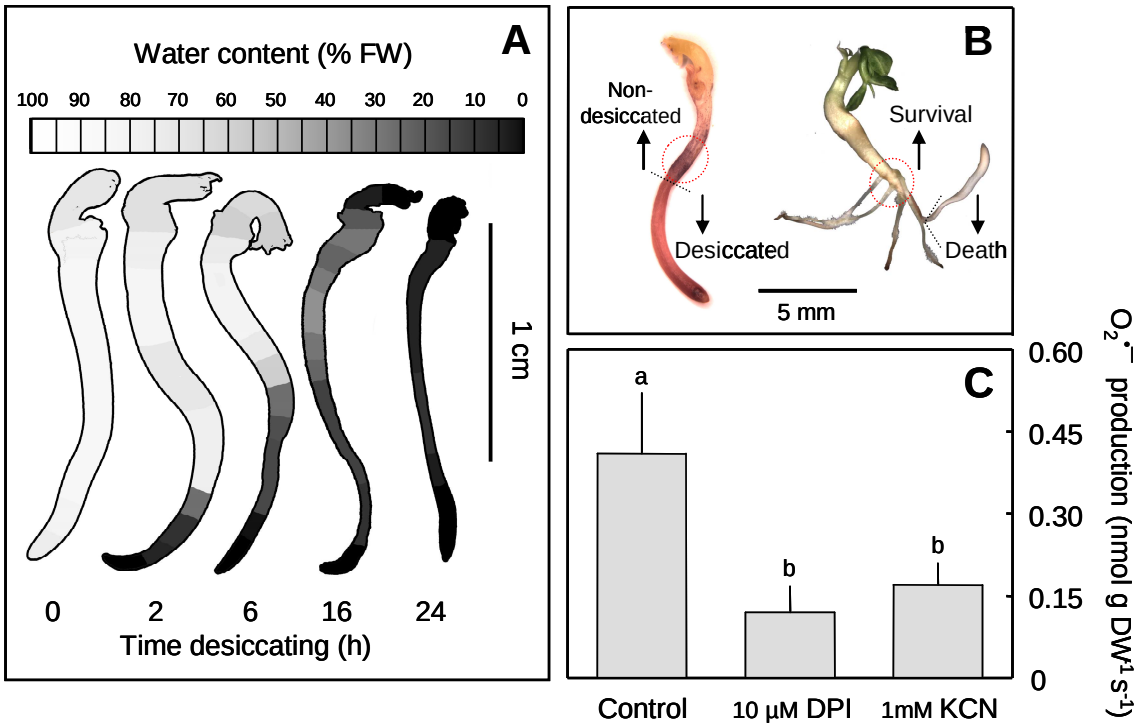


Figure 5.6 Water loss during seedling axis desiccation over silica gel (3 % RH) and investigations into the nature of the enzymes involved with $O_2^{\bullet-}$ production in the surviving section of mildly desiccated *Pisum sativum* seedling axes. **[A]** Distribution of water loss in seedling axes following 0, 2, 6, 16 and 24 h of desiccation. **[B]** Left image shows production of $O_2^{\bullet-}$, as indicated by purple staining, visualised after incubating 2-h desiccated axes in 5 mM NBT for 5 min. Note the production of $O_2^{\bullet-}$ just above the desiccated section highlighted by the red dashed circle. Right image shows that the desiccated section of the radicle died, but secondary root growth, as observed here after 21 d of culturing, occurred in the same region of enhanced $O_2^{\bullet-}$ production. **[C]** After separation from the desiccated part, the viable section of seedling axes was further explored by measuring $O_2^{\bullet-}$ production in the presence of NAD(P)H oxidase and ECPOX inhibitors (DPI) and (KCN), respectively, (means + SE, n = 4 reps of 5 seedling axes).

meristem was lost, but viability had been maintained seedling axes with secondary root growth occurring from the non-desiccated section. The viable non-desiccated section was responsible for 47 % of the total $O_2^{\bullet-}$ produced by 2 h desiccated seedling axes (n = 5 replicates of 5 seedling axes split into desiccated and non-desiccated parts). The production of $O_2^{\bullet-}$ was concentrated just above the desiccated section of the radicle (Fig. 5.6B). Further investigation into the mechanisms of $O_2^{\bullet-}$ production in the viable section of the $O_2^{\bullet-}$radicle revealed that at least two enzymes were involved. Inhibiting ECPOX or NAD(P)H oxidases significantly ($P < 0.05$) decreased $O_2^{\bullet-}$ production (Fig. 5.6C).

After 56 h of imbibition seedling axes were at different developmental stages, as indicated by their different radicle lengths (Fig. 5.2D; Fig. 5.4A). Desiccating seedling axes with radicle lengths of 14.9 ± 1.5 mm, 9.3 ± 0.9 mm or 3.4 ± 0.5 mm for 2 h over silica (3 % RH) resulted in WCs of 67.1 ± 3.6 %, 59.7 ± 2.4 % or 43.3 ± 4.0 %, respectively (Fig. 5.7A). Radicles at *c.* 15 mm in length were showing the first signs of root hair growth (not shown). Despite the lower WC following 2 h of desiccation, a greater number of seedling axes with shorter radicles grew shoots (Fig. 5.7B) and secondary roots (Fig. 5.7C) than longer seedling axes, as measured after 21 d in culture. However, improvements in both shoot viability and root growth of seedling axes with longer radicles that had been desiccated for 2 h could be gained by a 30 min treatment with 10 mM H_2O_2, compared to soaking in H_2O (Fig. 5.7B; Fig. 5.7C). A clear correlation between seedling length and H_2O_2 consumption by 2 h-desiccated axes was observed, with longer seedling axes breaking down a greater amount of H_2O_2 within 30 min than shorter seedling axes (Fig. 5.7A).

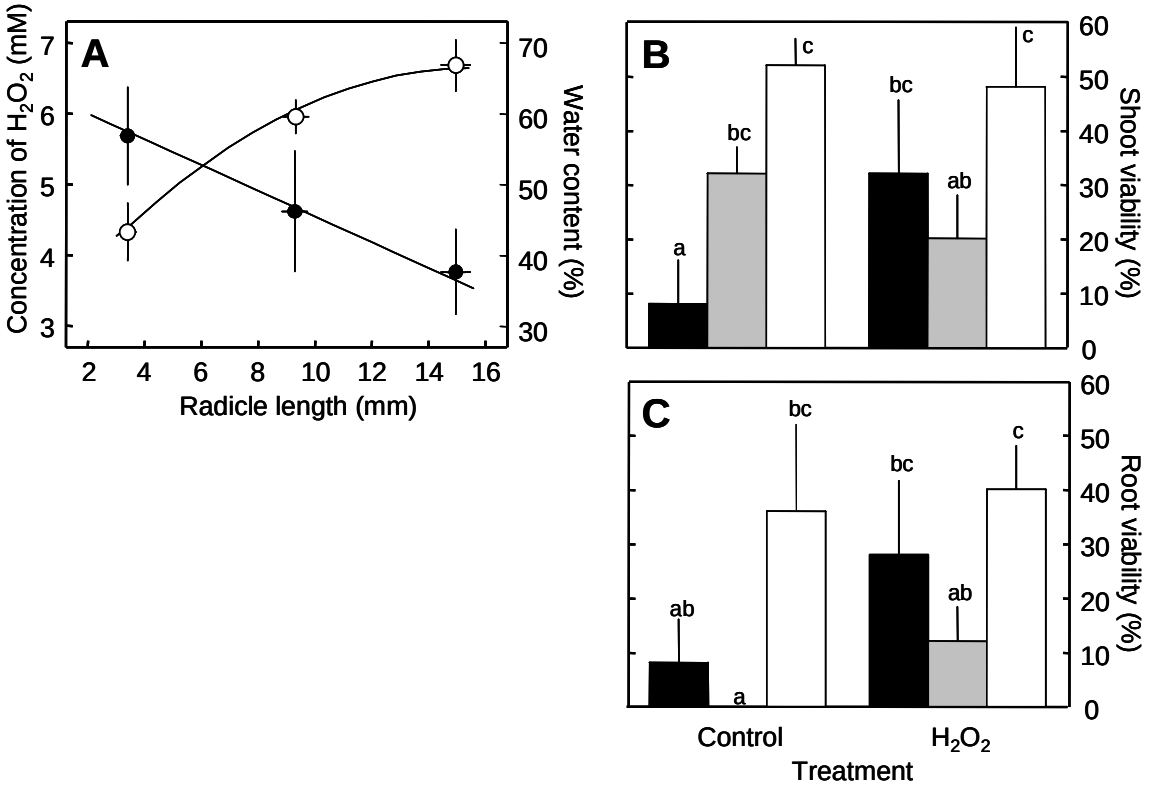


Figure 5.7 Viability of *Pisum sativum* seedling axes following mild desiccation as affected by axis length and H_2O_2 treatment. Seedling axes were split into groups according to radicle length, and then desiccated over silica gel (3 % RH) for 2 h. **[A]** Following desiccation, axes were weighed for WC measurements (open symbols, right y axis) before seedling axes were incubated in 10 mM H_2O_2 for 30 minutes. The concentration of H_2O_2 remaining in the 5 ml incubation solution was measured by the xylenol orange assay. 50 µl of incubation solution was added to 3 ml of xylenol orange working reagent (closed symbols, left y axis), (means ± SE, n = 4 replicates of 10 seedling axes). Seedling axes desiccated for 2 h were treated in H_2O or 10 mM H_2O_2 for 30 minutes to test the effect on viability. **[B]** Shoot and **[C]** root viability, as measured by axes that developed green shoots or secondary roots, respectively, is shown after 21 d of culturing at 25 ^{o}C. Black, grey and white bars represent seedling axes with radicle lengths of 16.4 ± 0.9 mm, 9.0 ± 0.5 mm or 2.6 ± 0.3 mm, respectively (means + SE, n = 5 replicates of 5 seedling axes).

5.3 Discussion

5.3.1 The dual nature of ROS

The ageing of *P. satium* seeds is associated with an oxidation of GSH, the most abundant water-soluble antioxidant (Kranner *et al*., 2006). With antioxidants becoming depleted, unabated production of intracellular ROS can initiate cellular damage to organelles, for example lipid peroxidation of phospholipid membranes (Varghese and Naithani, 2002; Goel *et al*., 2003). In Figure 5.1, it was observed that seed ageing led to reduced vigour likely due to the time it takes for seeds to regain a healthy cellular redox homeostasis and to repair damaged structures, such as phospholipid membranes. Ageing seed for 23 d decreased viability to 77 % and increased the leakage of electrolytes, a typical feature of damaged cell membranes (Sattler *et al*., 2004). Ageing seeds for 102 d, sufficient time for all seeds to lose viability, led to further membrane leakiness, highlighting the correlation of viability loss and membrane deterioration (Fig. 5.1B). Oxidative stress is one of the deleterious effects of ROS, causing seed ageing, but is only just one aspect of the nature of ROS within plants (Foyer and Noctor, 2005; Hallilwell, 2006), as discussed below.

Plant infection sites show localised rapid bursts of ROS production (Thordal-Christensen *et al*., 1997), a common feature of the plant defence response to pathogen attack (Baker and Orlandi, 1995; Bolwell *et al*, 2002). High concentrations of ROS can be directly toxic to pathogens and can act as a first line of defence (Murphy *et al*., 1998). The imbibition of *P. sativum* seeds was accompanied by an immediate oxidative burst (Fig. 5.1C). However, if seeds were re-desiccated and then rehydrated the immediate production of ROS did not return. In the field it is probable that seeds undergo several wetting and drying cycles before germination and a single sterilising event is unlikely to be sufficient to maintain seeds pathogen-free. Therefore, the biological significance is unclear. Unfortunately, the mechanism of the initial oxidative burst was unresolved as neither ECPOX nor NAD(P)H

oxidase inhibitors had any effect (Table 5.1). Intriguingly, the initial oxidative burst did not involve $O_2^{\bullet-}$, but the reactivity with epinephrine suggests that radical species were produced. Lipoxygenases, which metabolise poly-unsaturated fatty acids via radical intermediates to lipid peroxides, have been attributed to defending peanut seeds against seed-infecting fungi (Burow *et al.*, 2000). Furthermore, lipid peroxides from LOX activity may also account for activity detected by xylenol orange, as used for measuring H_2O_2 (Fig. 5.2C), but at this stage a LOX-mediated initial oxidative burst can only be tentatively proposed.

The initial oxidative burst declined in aged seeds (Fig. 5.1C), possibly due to enzyme inactivation, potentially leaving seedlings more vulnerable to infection. Between the initial burst and germination seeds produced very limited amounts of extracellular ROS. Only around the time when half of the seeds had germinated did the production of $O_2^{\bullet-}$ by non-aged seeds increase (Fig. 5.1C). The decline of $O_2^{\bullet-}$ production by seedlings from aged seeds is indicative of a link to viability losses. Measurements were made of seed populations, which after ageing included non-viable seeds that would have lowered rates of $O_2^{\bullet-}$ production. However, it is possible that seedlings from aged seeds may also be deficient in $O_2^{\bullet-}$-producing capabilities, as similar declines in activity have been identified in aged potato tubers (Kumar and Knowles, 2003). Nonetheless, the delay in production of extracellular $O_2^{\bullet-}$ by aged seedlings suggests that its control is under strict regulation associated with specific developmental stages of the seedling.

5.3.2 Physiological roles for ROS and ECPOX activity

The production of ROS may in part be related to resisting pathogens. Seeds were briefly dipped in ethanol and surface sterilised, which lowered seed mortality during germination, but conditions were not sterile. Considering the localised cell wall reinforcement that occurs following infection (Bradley *et al.*, 1992; Bernards *et al.*, 2004; Almagro *et al.*, 2009), newly developed radicles deficient in the protection afforded by lignin (Tokunaga *et al.*, 2009) suggests

that seedlings are particularly susceptible to pathogen infections. The patchy NBT staining along the exposed radicle indicates that $O_2^{\bullet -}$ is being produced along its entire length, and the random patterning may relate to various infection sites. ECPOX have a notorious role in plant pathogen resistance (Bradley *et al*., 1992; Bolwell *et al*., 1995; Otte and Barz, 1996; Bindschedler *et al*., 2006) and measurements of $O_2^{\bullet -}$ production in the presence of the ECPOX inhibitor NaN_3 identified its participation in *P. sativum* seedlings (Table 5.2).

The contribution of ECPOX in producing ROS is an important component of the plant response to pathogen challenge (Bolwell *et al*., 1995). For example, by inhibiting an ECPOX that contributes to the oxidative burst, Arabidopsis plants were more susceptible to pathogen challenges and had reduced survival (Bindschedler *et al*., 2006). Increases in cell surface ECPOX activity was detected in seedlings with radicle lengths greater than 10 mm (Fig. 5.3B), the same stage when an increase in extracellular $O_2^{\bullet -}$ production was observed (Fig. 5.2D). However, the paradoxical functions of ECPOX (Passardi *et al*., 2005) can lead to separate conclusions to the co-occurrence of $O_2^{\bullet -}$ production and ECPOX activity. On the one hand, ECPOX contribute to the production of $O_2^{\bullet -}$ in developing *P. sativum* seedlings (Table 5.2), possibly as described in the pathways shown in Figure 4.1. On the other hand, ECPOX can utilise H_2O_2 from $O_2^{\bullet -}$ dismutation for the production of $^{\bullet}OH$ to cleave cell wall polysaccharide chains allowing cellular elongation (Schopfer, 2001). Interestingly, Liszkay *et al*. (2004) showed that by inhibiting $O_2^{\bullet -}$ production the synthesis of $^{\bullet}OH$ was retarded, which negatively affected root growth. Cellular elongation occurs in the area just behind the radicle meristem (Scheres *et al*., 2002), which in *P. sativum* was associated with NBT reduction (Fig 5.2B), similar to the roots of Arabidopsis (Dunand *et al*., 2007) and maize (Liszkay *et al*., 2004). Within this continually developing region of the radicle, the extracellular production of $O_2^{\bullet -}$ is suggested to be utilised by ECPOX for cellular extension.

The roles of ECPOX in, for example, pathogen resistance and cellular extension, helps to explain why seedling axes expressed much higher ECPOX activity in comparison to the cotyledons and seed coats (Table 5.3). Furthermore, increases in $O_2^{\bullet-}$ production were tightly associated with radicle elongation (Fig 5.2D), without the seed coat contributing beyond the initial burst (Fig 5.2A). Therefore, the seedling axis is suggested to be responsible for the vast majority of $O_2^{\bullet-}$ production following germination. In 48 h imbibed seedlings $O_2^{\bullet-}$ was produced at a rate of 25 pmol g DW^{-1} s^{-1}, but at this stage the DW of an axis is around 60 times lighter than the DW including cotyledons. Therefore, $O_2^{\bullet-}$ production on the seedling surface may actually be in the region of 1.5 nmol g DW^{-1} s^{-1}, and even higher in specific regions (Fig 5.2B), which could affect the interpretation of mechanisms of how $O_2^{\bullet-}$ is produced.

Extracellular enzymes from the seedling axis potentially involved in $O_2^{\bullet-}$ production could be identified using electrophoresis in combination with in-gel activity staining. Extracellular enzymes capable of reducing NBT were only observed in seedlings with radicles of 11 ± 1 mm or greater (Fig. 5.3C), in agreement with $O_2^{\bullet-}$ production from the root surfaces not occurring in recently germinated seeds (Fig. 5.2D). In seedlings with radicle lengths greater than 15 mm, several extracellular enzymes are capable of NBT reduction ($O_2^{\bullet-}$ production), most notably one that was *c.* 70 kD in size (Fig. 5.3C). Although typically ECPOX have molecular masses between 28 and 60 kD (Hiraga *et al.*, 2001), a 72 kD isoform has been isolated from the apoplast of onion roots (Córdoba-Pedregosa *et al.*, 2003). Interestingly, this ECPOX isoform was predominantly found in the root elongation zone (Córdoba-Pedregosa *et al.*, 2003), which in *P. sativum* radicles is associated with enhanced $O_2^{\bullet-}$ production (Fig 5.2B).

Figure 5.3D shows that the *c.* 200 kD ECPOX could be stimulated to produce $O_2^{\bullet-}$ using cysteine, ferulic acid, NADH or NADPH in the presence of H_2O_2. The location of the activity stain may be misleading regarding the size of the enzyme as 200 kD is very large for an ECPOX. The enzyme may have

formed an oligomer complex or might not have migrated through the gel, although clearly it was capable of breaking down H_2O_2 (Fig. 5.3A) or producing $O_2^{\bullet-}$ (Fig. 5.3D). However, the contribution of H_2O_2 to $O_2^{\bullet-}$ production in *P. sativum* seedlings is not clear as only low levels of extracellular H_2O_2 were detected during germination (Fig. 5.2C), and some enzymes could reduce NBT in the presence of only NADH (Fig. 5.3C). In Arabidopsis, the production of $O_2^{\bullet-}$ that is essential in the signalling and development for normal root growth is provided by NAD(P)H oxidase (Foreman *et al.*, 2003). Inhibiting either ECPOX or NAD(P)H oxidase in *P. sativum* seedlings inhibited $O_2^{\bullet-}$ production (Table 5.2). The specificity of enzyme inhibitors was questioned in chapter 4, but if both enzymes do contribute to the production of $O_2^{\bullet-}$, it is possible that each enzyme is active in different root sections. Therefore I suggest that in the infected sections of the radicle, ECPOX produce $O_2^{\bullet-}$ for pathogen resistance whereas in the elongation zone, NAD(P)H oxidase may contribute to $O_2^{\bullet-}$ production for developmental signalling and ECPOX-mediated $^{\bullet}OH$ production. Taken together, results suggest that the generation of $O_2^{\bullet-}$ and H_2O_2 during *P. sativum* seedling development is under a strict regulatory control, in which apoplastic ECPOX play a renowned role.

5.3.3 Extracellular $O_2^{\bullet-}$ production by desiccating seedlings

Following germination *P. sativum* seeds lose desiccation tolerance (Reisdorph and Koster, 1999). Here, 90 % of seeds imbibed for 6 h remained desiccation tolerant, but all seeds imbibed for 56 h were sensitive to desiccation (Fig. 5.5B). Hence, seedling axes expressed a different response to desiccation than embryonic axes, as indicated by the differences in extracellular $O_2^{\bullet-}$ production. As hypothesised, no extracellular $O_2^{\bullet-}$ was observed when desiccating embryonic axes from 72 to 10 % WC (Fig. 5.5B; Fig. 5.5D). The results obtained from desiccated seedling axes are more complex because $O_2^{\bullet-}$ is produced during radicle growth (Fig. 5.1A). An increase in extracellular $O_2^{\bullet-}$ was observed within the first few hours of desiccation (Fig. 5.5C) similar to the results of recalcitrant *C. sativa* embryonic

axes. However, there are major differences in the physiology of *C. sativa* embryonic axes and *P. sativum* seedling axes that need addressing. The pre-desiccation WC of *P. sativum* seedling axes was 30 % greater than that of *C. sativa* embryonic axes (Fig. 3.1A; Fig. 5.4A). Also, the physiology associated with the rapid growth of seedlings renders them prone to even slight desiccation and unlike *C. sativa* axes, maximum rates of $O_2^{\bullet-}$ production peaked when all viability had been lost rather than before (Fig. 3.9C; Fig. 5.5C). Seedling axes from orthodox seeds are unlike recalcitrant seeds in many developmental aspects confounding a comparison of their individual responses to desiccation. Nonetheless, during non-critical mild desiccation *P. sativum* seedlings exhibited enhanced extracellular $O_2^{\bullet-}$ production, suggesting a potential role in desiccation response, similar as found in recalcitrant *C. sativa* embryonic axes.

Following temporary drought that kills the radicle meristem (Fig. 5.6A) seedlings have mechanisms that permit survival (Farrant *et al*., 2004). Reactive oxygen species contribute to signalling events that can initiate programme cell death (PCD), which has been identified to have occurred in desiccation stressed seedlings (Faria *et al*., 2005). Increasing desiccation time increased the production of $O_2^{\bullet-}$ (Fig. 5.5C), possibly from PCD activity in the section of the radicle that lost viability. However, rapid desiccation using silica gel may have provided insufficient time for the execution of PCD and random events associated with necrosis may have contributed to $O_2^{\bullet-}$ production. Therefore, attention was focused towards the viable section of seedling axes. The production of $O_2^{\bullet-}$ was enhanced in the region just above the desiccated section where new root growth was observed (Fig. 5.6B). Here, both NAD(P)H oxidases and ECPOX were partially responsible for desiccation-induced $O_2^{\bullet-}$ production (Fig. 5.6C). A mechanism to integrate both enzymes in the production of ROS was described by Bindschedler *et al*. (2006) where ECPOX provides the immediate source of ROS, which triggers a secondary response by NAD(P)H oxidase. As ECPOX-mediated $O_2^{\bullet-}$ production may well

be dependent upon the availability of H_2O_2 (Fig. 4.1), treating seedlings with H_2O_2 could influence the response to desiccation stress.

5.3.4 Treating mildly desiccated seedlings with H_2O_2 improved viability

After 2 h of desiccation, smaller seedling axes achieved better survival than longer axes, possibly because they had maintained residual mechanisms of desiccation-tolerance from seeds (Buitink *et al.*, 2003; Faria *et al.*, 2005). Less than 10 % of seedlings with 16 mm radicles were viable following 2 h of desiccation, but this was improved by a 30 min post-desiccation treatment with 10 mM H_2O_2 (Fig. 5.7B and Fig. 5.7C). The ability of longer axes in being able to utilise the H_2O_2 could be derived from their greater ECPOX activity (Fig. 5.3B). Interestingly, in strawberry cultures increases in H_2O_2 coincided with the emergence of bud primordium (Tian *et al.*, 2003), while H_2O_2 induced and promoted somatic embryogenesis in tomato callus (Cui *et al.*, 1999). However, AA is also implicated in cell proliferation (De Tullio *et al.*, 1999; Arrigoni and De Tullio, 2002; Córboda-Pedregosa *et al.*, 2004) and cell elongation (Hidalgo *et al.*, 1989; González-Reyes *et al.*, 1994), suggesting a complex interaction between prooxidants and antioxidants during growth.

5.3.5 Summary

In summary, this work highlights the dual nature to ROS. Oxidative stress leads to seed ageing through processes such as membrane damage, but ROS may also facilitate seedling survival. For example, the initial oxidative burst may sterilise the seed, although further work is required to elucidate its mechanisms and functionality. Following germination, the tight relationship between ECPOX activity and extracellular $O_2^{\bullet-}$ production is indicative of more than just a casual relationship, with both likely to participate in pathogen defence and cell growth. Of all seedling organs, the axis was particularly rich in ECPOX activity. During radicle extension an increasing number of $O_2^{\bullet-}$-producing enzymes were synthesised. The production of $O_2^{\bullet-}$ was shown to be carefully regulated, as revealed by the delay in production by aged seed.

Seedlings were sensitive to desiccation and responded by increasing their rates of extracellular $O_2^{\bullet-}$ production, similar to recalcitrant *C. sativa* embryonic axes. In 2 h desiccated seedlings, the sites of new adventitious root growth coincided with the area of enhanced $O_2^{\bullet-}$ production, indicative of a role for $O_2^{\bullet-}$ in the stress response for regeneration. Furthermore, improvements in the establishment of root and shoot meristems of 2 h desiccated seedling axes were observed following H_2O_2 treatment. This suggests that ROS may facilitate the survival of stress by mitigating the damage following non-lethal desiccation, on top of their importantance in normal growth and development, as has been previously published.

Chapter 6 Mechanisms of reactive oxygen species production following the wounding of *Pisum sativum* seedlings

6.1 Introduction

Herbivore grazing and physical stress can rupture plant surfaces providing an entrance to pathogens. In response, plant defence systems become activated, including an immediate production of ROS, referred to as the 'oxidative burst' (Legendre *et al*., 1993; de Bruxelles and Roberts, 2001). Upon injury, small oligosaccharide fragments are released from the plant cell walls, which elicit defence responses (Côté and Hahn, 1994), including the oxidative burst (Legendre *et al*., 1993; Stennis *et al*., 1998). At the wound site, ROS may directly attack potential pathogens (Murphy *et al*., 1998), rebuild and reinforce cell walls (Bradley *et al*., 1992; Pothika *et al*., 1999), as well as participate in localised and systemic signalling (Alvarez *et* al., 1998; Seo *et al*., 1999; de Bruxelles and Roberts, 2001). Plants have multiple enzymes capable of producing ROS, such as ECPOX, AOX, NAD(P)H oxidase and LOX (Doke 1985; Kanofsky and Axlerod, 1986; Allan and Fluhr, 1997; Bolwell *et al*., 2002), but their contribution to the wound-induced oxidative burst in *P. sativum* seedlings is not fully understood.

In chapter 5, it was shown that ECPOX activity in *P. sativum* seedlings increased during radicle elongation (Fig. 5.3B) and was involved in $O_2^{\bullet -}$ production (Table 5.2). It has been suggested that ECPOX requires H_2O_2 for the production of $O_2^{\bullet -}$ (Fig. 4.1). Copper containing AOX, the most abundantly soluble extracellular protein in *P. sativum* seedlings (Federico and Angelini, 1991), produces H_2O_2 in the presence of polyamines, most efficiently with putrescine (Pietrangeli *et al*., 2007). Both AOX and ECPOX are found in lignifying sections of lentil and chickpea roots, where AOX-derived H_2O_2 is utilised for cell wall stiffening by ECPOX (Angelini *et al*., 1990; Rea *et al*., 1998). Therefore, it is possible that both enzymes work together in producing

$O_2^{\bullet-}$. Only low levels of extracellular H_2O_2 were detected during *P. sativum* seedling growth (Fig. 5.1D), but wounding is likely to induce production (Orozco-Cárdenas and Ryan, 1999; Chandra *et al*., 2000; Le Deunff *et al*., 2004). Considering the ECPOX-mediated scheme of $O_2^{\bullet-}$ production in Figure 4.1, electron donors are required for $O_2^{\bullet-}$ production. Despite the identification of ECPOX in wounding-induced (Minibayeva *et al*., 2003; 2009) and pathogen-induced (Bolwell *et al*., 1995; Bindschedler *et al*., 2006) ROS production, the identity of ECPOX reductants remains elusive.

Early attempts to characterise reductants used by ECPOX to produce ROS have led to the proposal of electron donors that only have limited physiological relevance (Gross *et al*., 1977; Halliwell, 1978; Pichorner *et al*., 1992). For example, NADH and cysteine work as reductants *invitro* in the presence of Mn^{2+} and phenolic compounds, (Gross *et al*., 1977; Halliwell, 1978; Pichorner *et al*., 1992), but the co-occurrence of these substrates in the apoplastic environment is questionable. Minibayeva *et al*. (2001) showed that di- and tri- carbonic acids stimulated KCN-sensitive $O_2^{\bullet-}$ production in wounded wheat roots, but no information is available on their presence at wound sites. Bolwell *et al*. (2002) identified a release of fatty acids during the pathogen-induced oxidative burst of plant cell cultures, but it was inconclusive whether they participated in the production of ROS. In chapter 3, it was shown that ionically bound cell wall enzymes of *C. sativa* could be rapidly stimulated to produce $O_2^{\bullet-}$ in the presence of the lignin precursor ferulic acid. It is also possible that ECPOX can produce $O_2^{\bullet-}$ in the presence of H_2O_2 via compound III (Baker *et al*., 2000; Hiner *et al*., 2001), questioning whether ECPOX-mediated $O_2^{\bullet-}$ production actually requires other substrates than H_2O_2, which in the presence of peroxidases can act as an oxidant and reductant (Mika *et al*., 2004).

In this chapter, the oxidative burst following the wounding of *P. sativum* seedlings is characterised, testing the hypothesis that AOX will produce the H_2O_2 required for ECPOX-mediated $O_2^{\bullet-}$ production. Measurements of $O_2^{\bullet-}$ and H_2O_2 were made immediately following wounding

and in the presence of enzyme inhibitors. Proteins were also extracted from cell walls for further *invitro* studies to identify specific enzymes capable of producing $O_2^{\bullet -}$. Potential substrates leaked from wounded seedlings were tested for their capability to induce the production of ROS, providing *invivo* evidence of particular enzyme-mediated ROS production.

6.2 Results

6.2.1 Characterising the wounding-induced oxidative burst

Following the excision of *P. sativum* seedling axes an immediate catalase-sensitive oxidation of xylenol orange occurred in the presence of the incubation solution, suggesting a release of H_2O_2 as a consequence of wounding (Fig. 6.1A). Subtracting non-specific activity revealed that H_2O_2 was produced at a rate of 0.21 ± 0.05 nmol g DW^{-1} s^{-1} for the first 5 min, with production falling to 0.06 ± 0.02 nmol g DW^{-1} s^{-1} between 5 to 10 min and to even lower rates thereafter. The addition of SOD had no significant ($P < 0.05$) affect upon the amount of H_2O_2 produced (Fig. 6.1A). The rate of adrenochrome formation fell over 20 min post wounding, but actual production of $O_2^{\bullet-}$ fluctuated around 1 nmol g DW^{-1} s^{-1} (Fig. 6.1B). Further results (data not shown) revealed that $O_2^{\bullet-}$ production from wounded *P. sativum* seedling axes declined to background levels observed in non-wounded seedling axes within 1 h. Interestingly, the addition of catalase reduced the amount of $O_2^{\bullet-}$ produced by 72 % (Fig. 6.1B), indicative of an essential role for H_2O_2 in $O_2^{\bullet-}$ production. The peak amount of H_2O_2 detected in the incubation solution was observed 8 min after wounding, with levels falling thereafter (Fig. 6.1C). Adding exogenous H_2O_2 increased the amount of $O_2^{\bullet-}$ production by wounded *P. sativum* seedling axes (Fig. 6.1D).

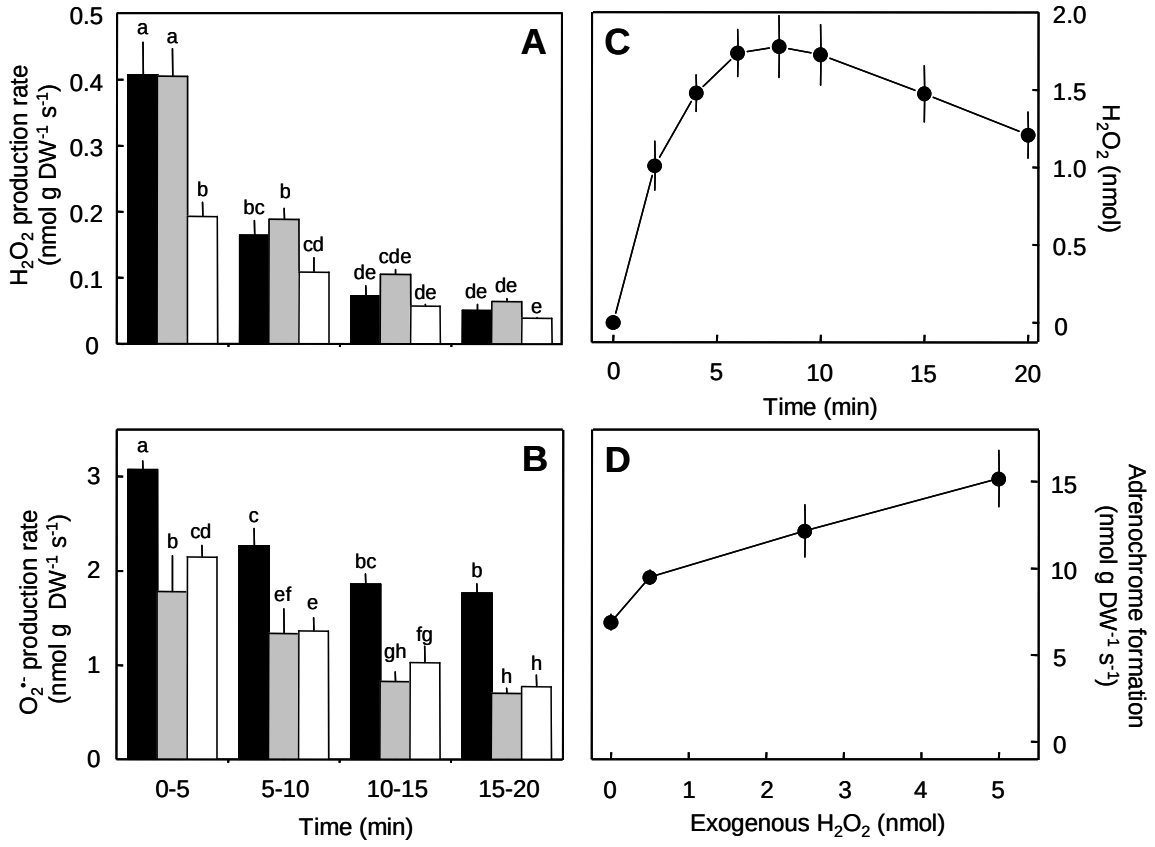


Figure 6.1 The oxidative burst following the wounding of *Pisum sativum* seedlings and the effect of exogenous H_2O_2 upon $O_2^{\bullet-}$ production. Wounding was initiated in seeds imbibed for 56 h by excising the seedling axes from cotyledons and placing the axes in 5 ml of incubation solution for up to 20 min. **[A]** H_2O_2 production was quantified using the xylenol orange method, with 0.5 ml of incubation solution removed for measurement every 5 min. **[B]** Every 5 min $O_2^{\bullet-}$ production was quantified in 1.0 ml of incubation solution (returned after measurement) by measuring the oxidation of epinephrine to adrenochrome. In [A] and [B], black bars represent control. Measurements made in the presence of SOD (200 units ml^{-1}) or catalase (250 units ml^{-1}) are represented by the grey and white bars, respectively. **[C]** Measurements of the amount of H_2O_2 in the incubation solution containing wounded seedling axes. **[D]** Wounded seedling axes were incubated in various concentrations of exogenous H_2O_2 and measurements of $O_2^{\bullet-}$ production, assessed by adrenochrome formation, were taken after 5 min, (means + SE, [A] n = 3 replicates of 10 seedling axes, [B] n = 3 replicates of 5 seedling axes, [C] n = 4 replicates of 10 seedling axes and [D] n = 4 replicates of 5 seedling axes).

6.2.2 Contribution of enzymes to the wounding-induced oxidative burst

Rates of wounding-induced H_2O_2 production were most reduced by inhibiting AOX. During 10 min after wounding, incubation with 5.0 mM 2-bromoethylamine lowered measurements to those observed in the presence of catalase (Fig. 6.2A). Furthermore, the production of $O_2^{\bullet -}$ was also completely inhibited by eliminating the activity of AOX (Fig. 6.2B). Using DPI, a NAD(P)H oxidase inhibitor, production of H_2O_2 and $O_2^{\bullet -}$ was reduced by one third of the controls (Fig. 6.2A; Fig. 6.2B). Incubating wounded seedling axes with NaN_3, an ECPOX inhibitor, almost doubled the rate of H_2O_2 production (Fig. 6.2A) whereas the production of $O_2^{\bullet -}$ declined in a concentration dependent manner (Fig. 6.2B).

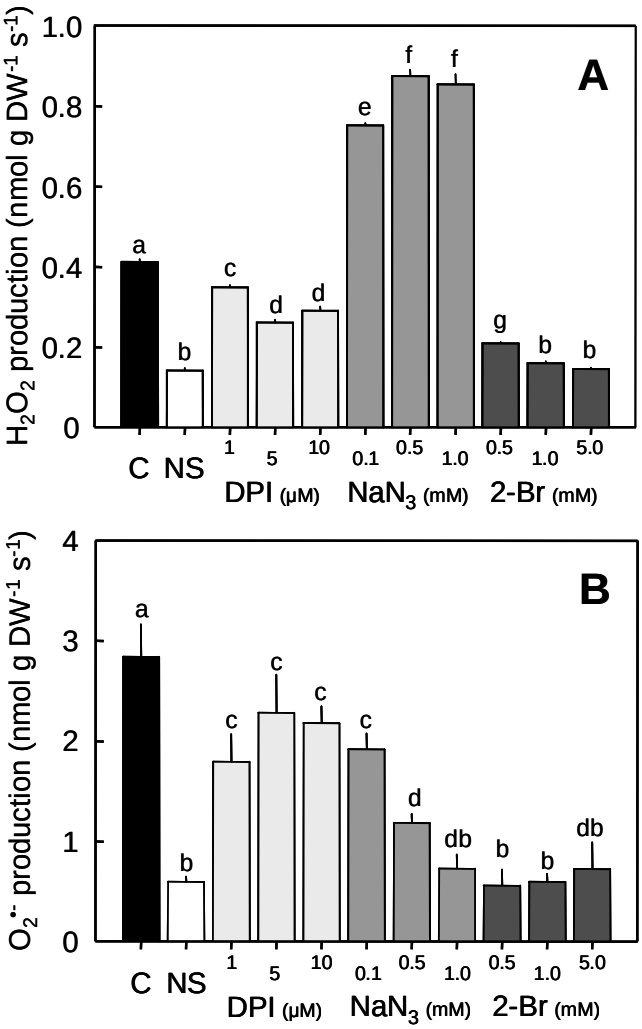


Figure 6.2 The effect of enzyme inhibitors on the wounding-induced oxidative burst of *Pisum sativum* seedlings. Production of **[A]** H_2O_2 and **[B]** $O_2^{\bullet -}$ were measured after 10 min of incubation. Black bars represent controls and white bars indicate non-specific activity as measured in the presence of 250 units ml^{-1} catalase or 200 units ml^{-1} SOD for [A] and [B], respectively. In both figures NAD(P)H oxidases, ECPOX and AOX inhibitors are indicated by light, medium and dark grey bars, respectively. Bars labeled from left to right: C, control; NS, non-specific activity; DPI (diphenyleneiodonium) 1, 5 or 10 µM; NaN_3 (sodium azide) 0.1, 0.5 and 1.0 mM; 2-Br (2-bromoethylamine) 0.5, 1.0 and 5.0 mM (means + SE, n = 4 replicates of 5 seedling axes).

An increase in AOX occurred during seedling development (Fig. 6.3A). The in-gel stained band represented a size of 150 kD, typical of *P. sativum* AOX dimers (Padiglia *et al.*, 1991). Wounded seedlings produced a greater amount of H_2O_2 in the presence of exogenous putrescine confirming the presence of AOX. Significant ($P < 0.05$) increases were observed at putrescine concentrations up to 0.1 mM in the incubation solution (Fig. 6.3B). By inhibiting AOX, the amount of $O_2^{\bullet -}$ production during the first 10 min after wounding was reduced by 7.5 times (Fig. 6.3C). Between 20 – 30 min after wounding, no significant differences in $O_2^{\bullet -}$ production occurred in the presence or absence of the AOX inhibitor.

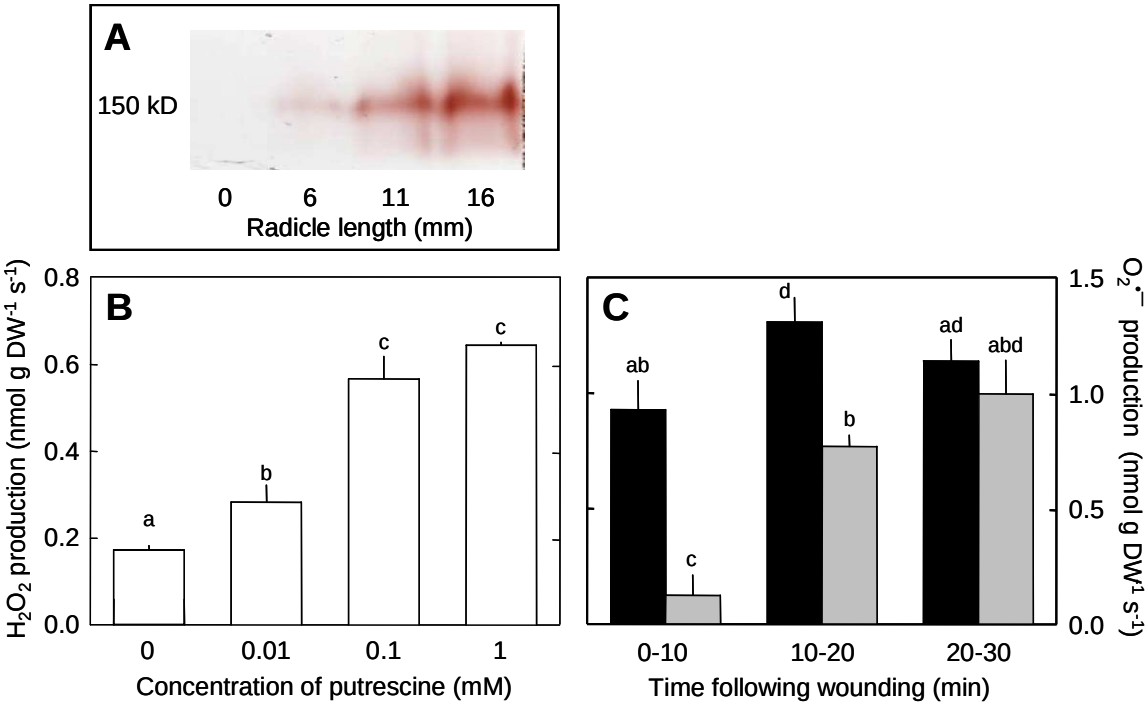


Figure 6.3 Effect on H_2O_2 and $O_2^{\bullet -}$ production by influencing AOX activity. **[A]** Relative abundance of extracellular AOX at various developmental stages of seedlings, as assessed by the seedling radicle lengths used for enzyme extraction. Bands were visualised in 50 mM K-phosphate buffer at pH 7.0 in the presence of putrescine, which is used by AOX to produce the H_2O_2 needed for the HRPOX-coupled reaction with guaiacol. The single band on the gel corresponded to 150 kD. **[B]** H_2O_2 production by wounded seedlings after 15 min incubation in the presence of various concentrations of exogenous putrescine. **[C]** Measurements of $O_2^{\bullet -}$ calculated after subtracting the non-specific activity recorded in the presence of 200 units ml^{-1} SOD. Black bars represent control and grey bars represent the production of $O_2^{\bullet -}$ in the presence of 1 mM of the AOX inhibitor 2-bromoethylamine (means ± SE, n = 4 replicates of 5 seedling axes for [B; C].

A cellular fractionation was conducted on seedling axes of various radicle lengths to further characterise ROS producing enzymes during seedling development. Extracellular proteins isolated in fractions B1, B2, B3 or B4 were loosely associated, bound by hydrophobic interactions, ionically bound or irremovably attached, respectively, to the cell wall. Table 6.1 shows the majority of extracellular AOX and ECPOX activity was in the B1 fraction. However, a quarter of ECPOX activity was associated with ionically bound proteins. In Figure 6.4, all removable cell wall enzymes (B1, B2 and B3) were fractionated together in one fraction and separated from the crude cellular fraction (C) and B4 fraction. Activity of AOX could not be detected in embryonic axes from seeds imbibed for only 3 h, whereas some ECPOX were present (Fig. 6.4). Following germination, the greatest enzyme activity was always in the C fraction. Here, the ECPOX activity was 2.5 times greater than AOX activity (Fig. 6.4A). There was an indication in seedling axes with radicle lengths of 16.4 mm that the amount of AOX and ECPOX in the C fraction was reaching its maximum (Fig. 6.4A). The activities of AOX and ECPOX in the B1 – B3 fraction increased almost identically during early seedling development. However, after seedlings had grown radicles longer than 9.0 ± 0.5 mm, the increase in ECPOX activity was greater (Fig. 6.4B).

Table 6.1 The distribution of ECPOX and AOX activities, given as percentages of total extracellular activity of each enzyme, in cell wall fractions of *Pisum sativum* seedling axes. Cell wall fractions: B1, proteins loosely associated to the cell wall; B2, proteins bound by hydrophobic interactions; B3, proteins bound by ionic bonds; B4, un-removable cell wall proteins. Fresh material was used for extractions and percentages and statistics were calculated after enzyme activities had been converted to μkat g FW^{-1}.

	Activity (%)	
	ECPOX	**AOX**
B1	56.4 ^{a}	61.1 ^{A}
B2	17.7 ^{c}	21.7 ^{B}
B3	25.1 ^{b}	00.7 ^{C}
B4	00.9 ^{d}	16.6 ^{B}

When using fresh material, defined as non-lyophilised seedling axes, almost all ECPOX could be removed from cell walls (Table 6.1). However, when using lyophilised seedling axes the removal of enzymes from the cell wall was restricted. For example, the ECPOX activities in the B4 fraction increased from 1 % to 8 % when using fresh and lyophilised material, respectively (Table 6.1; Fig. 6.4). Restrictions to extracting AOX from lyophilised material were also observed.

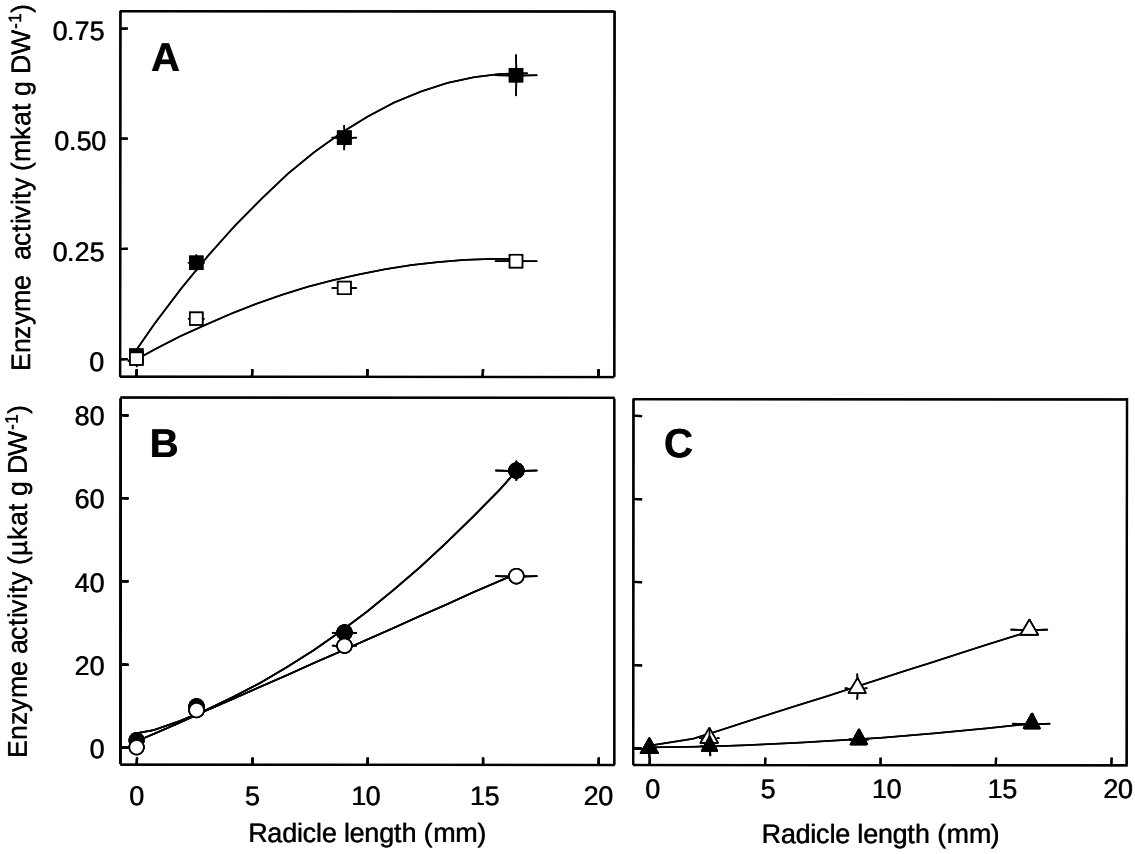


Figure 6.4 The metabolism and production of H_2O_2 by ECPOX and AOX, respectively, in various cell fractions of non-germinated *Pisum sativum* seeds and at the early stages of seedling development. Enzyme activity was measured by the H_2O_2-dependent oxidation of ABTS by POX. ECPOX activity (closed symbols) was measured in the presence of 10 mM H_2O_2. AOX activity (open symbols) was measured in the presence of 1 mM putrescine and 50 µg ml^{-1} horseradish-POX. Measurements were made of **[A]** C fraction, **[B]** B1 – B3 fraction and **[C]** B4 fraction. To isolate non-germinated embryonic axes (radicle length = 0), seeds were soaked in water for 3 h allowing sufficient water uptake for axis excision. Other seedling axes were isolated from seeds imbibed for 56 h of different developmental stages, as assessed by radicle length. Lyophilised material was used for all extractions (means ± SE, n = 3 replicates of 50 seedling axes using the same material for all three cellular fractionations).

To begin characterising extracellular enzymes involved in $O_2^{\bullet-}$ production, the four separately isolated cell wall fractions were incubated in the presence of epinephrine and various concentrations of NADH, cysteine and H_2O_2. Unexpectedly, some adrenochrome, the product of epinephrine oxidation by $O_2^{\bullet-}$ (Misra and Fridovich, 1972), was formed without any exogenous substrates (Fig. 6.5A; Fig. 6.5B). Increasing the concentration of H_2O_2 up to 0.1 mM, either in the presence or absence of NADH, led to the formation of 17 pmol g DW^{-1} s^{-1} of adrenochrome by the B1 fraction (Fig. 6.5A). Increasing the concentration of NADH without H_2O_2 led to small increases in adrenochrome formation, whereas cysteine inhibited the reaction regardless of the presence of H_2O_2 (Fig. 6.5A). Compared to B1, the B3 fraction produced slightly less adrenochrome in the presence of NADH in combination with H_2O_2, but cysteine had less of an inhibitory effect (Fig. 6.5B). The pattern in activity of fractions B2 and B4 were similar to B1 in the presence of all reagents, but with overall lower activity (data not shown). When the specificity of the assay to $O_2^{\bullet-}$ was tested with the B1 fraction, adrenochrome formation was insensitive to SOD and therefore not specific to $O_2^{\bullet-}$ (data not shown).

The ECPOX activity of fractions (Table 6.1) had a strong positive correlation to their ability to oxidise epinephrine in the presence of 100 µM H_2O_2 alone (Fig. 6.5C), showing that adrenochrome formation is likely to be dependent upon ECPOX activity. It also shows that fraction B3 forms more adrenochrome relative to ECPOX activity compared to the other fractions. Therefore, some of the adrenochrome formation by the B3 fraction in the presence of just H_2O_2 could be from $O_2^{\bullet-}$ production, which is explored later, while it is considered that the majority of adrenochrome formation by B1, B2 and B4 fractions is non-specific to $O_2^{\bullet-}$.

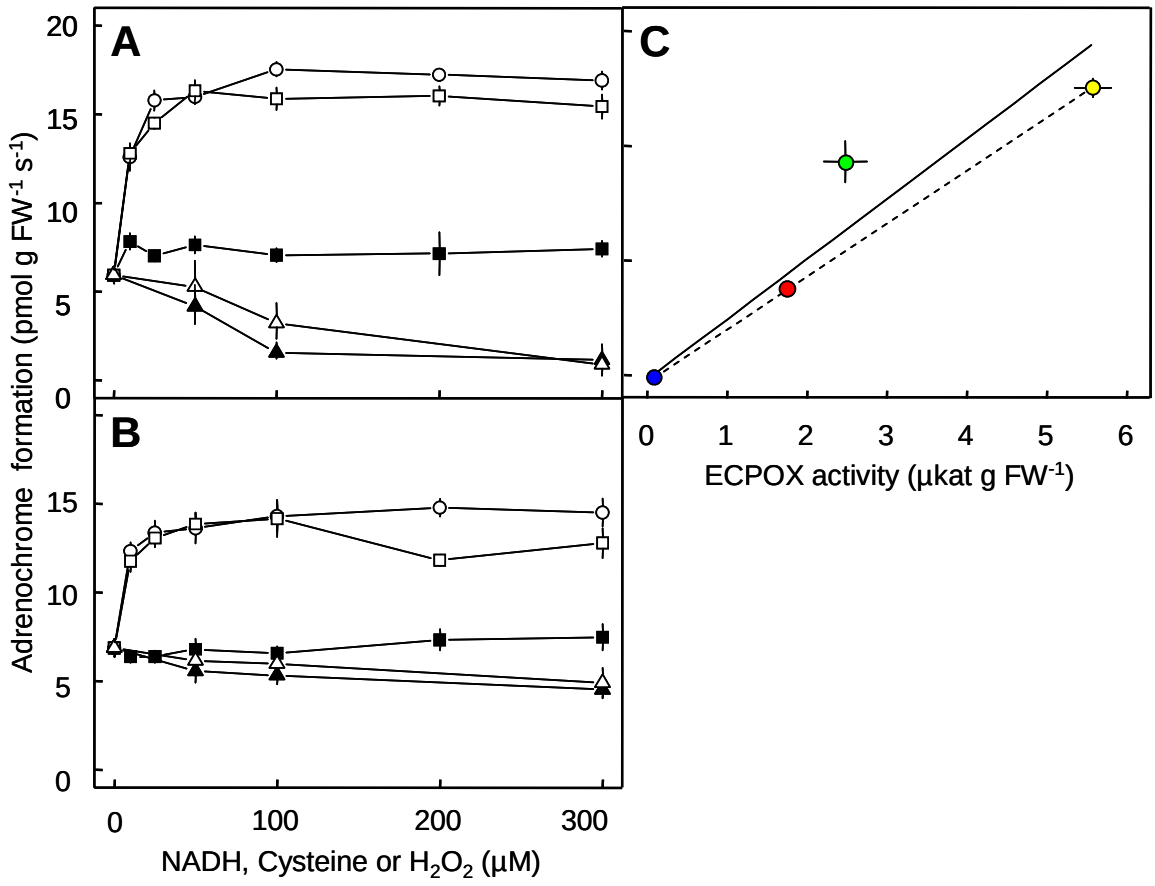


Figure 6.5 Characterisation of extracellular enzymes in *Pisum sativum* seedlings involved in $O_2^{\bullet-}$ production. **[A]** and **[B]** show fractions B1 and B3, respectively. Fractions were incubated in 50 mM phosphate buffer at pH 7.0 containing 1 mM epinephrine in the presence of various concentrations of NADH (closed squared), NADH and H_2O_2 (open squares), cysteine (closed triangles), cysteine and H_2O_2 (open triangles) or H_2O_2 only (open circles) for 10 minutes. When combined, NADH and H_2O_2 or cysteine and H_2O_2 were at the same concentration. **[C]** Scatter plot of ECPOX activity, assessed by the H_2O_2-dependent oxidation of ABTS (Table 6.1) against adrenochrome formation in the presence of 100 µM H_2O_2 of fractions B1, B2, B3 and B4, as represented by yellow, red, green and blue symbols, respectively. For definitions of B1 – B4 see table 6.1. The 'full' line of best fit considers all values and the 'dashed' line excludes data from fraction B3, (means ± SE, n = 4 replicates of 50 seedling axes. The same material was used for all fractionations).

Considering the results of Figure 6.5C, ionically bound enzymes were further investigated. Separating the B3 fraction with PAGE followed by NBT staining in the presence of 0.5 mM NADH identified five distinct bands (Fig. 6.6A) of enzymes capable of $O_2^{\bullet -}$ production (Fig. 6.6B). The sequencing of these bands identified two LOX isoforms and dihydrolipoyl dehydrogenase (Table 6.2).

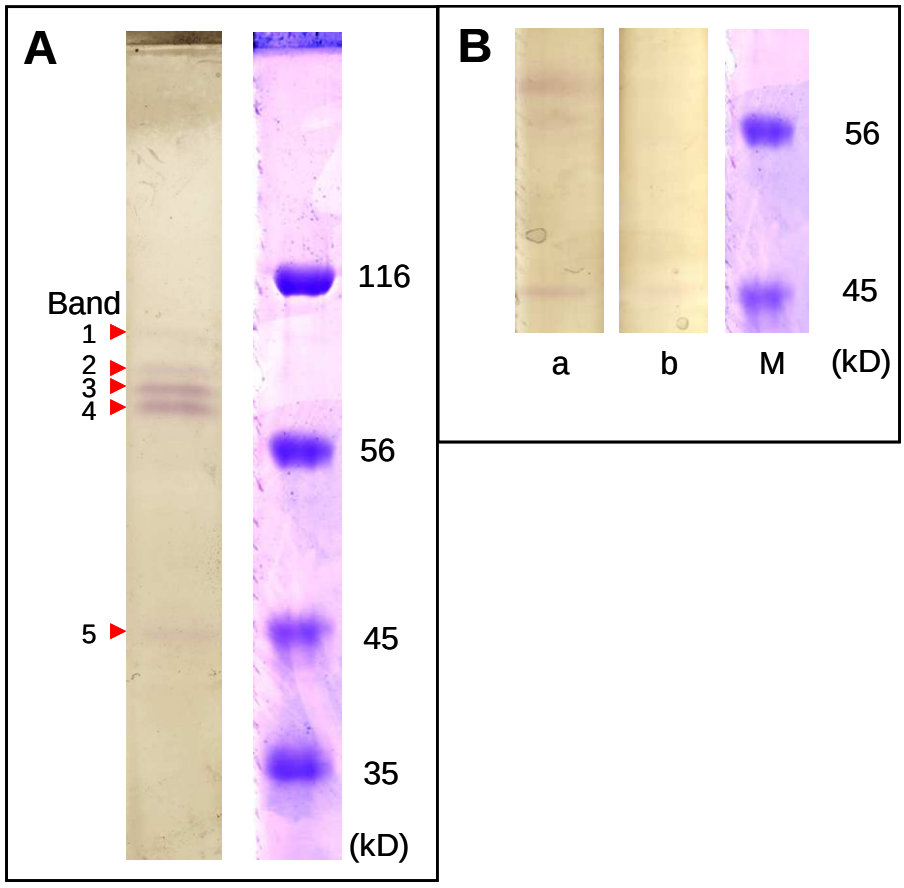


Figure 6.6 Ionically bound cell wall enzymes capable of producing $O_2^{\bullet -}$. Enzymes in the B3 fraction were electrophoretically separated and purple bands indicate $O_2^{\bullet -}$ production in the presence of 0.5 mM NADH and NBT. **[A]** Left gel; red arrows indicate the five bands of $O_2^{\bullet -}$ production. Right gel; the molecular mass marker. **[B]** Specificity of the stain to $O_2^{\bullet -}$ was confirmed, with lanes (a) and (b) representing in-gel staining in the absence and presence of 200 units ml^{-1} SOD, respectively. (M) shows the molecular mass marker.

Table 6.2 The identification of ionically bound *Pisum sativum* seedling enzymes capable of $O_2^{\bullet-}$ production courtesy of Dr Lucas Bowler (TCMR, University Sussex, UK). Purple bands, as shown in Fig. 6.6, were cut from the gel, digested, sequenced and identified using an annotated database (Swissprot) using the SEQUEST protein identification algorithm. All sequences were matched to proteins that had been previously sequenced in *P. sativum.* Each band contained up to 50 proteins and only redox active enzymes are provided below. The 'peptide coverage' shows the percentage of the total protein that was sequenced, thereby indicating its relative abundance in the purple NBT-stained band, as shown in Fig 6.7. The predicted complete enzyme size is also provided in kD.

Band	Protein identification	Complete enzyme size (kD)	Peptide coverage (%)
1	Seed LOX-2 Seed LOX-3	97 98	32 29
2	Dihydrolipoyl dehydrogenase Seed LOX-2 Seed LOX-3	53 97 98	22 20 16
3	Dihydrolipoyl dehydrogenase Seed LOX-2 Seed LOX-3	53 97 98	34 13 12
4	Dihydrolipoyl dehydrogenase	53	24
5	Dihydrolipoyl dehydrogenase	53	24

Some *invitro* measurements have shown that the presence of transition metals, such Mn^{2+}, enhances $O_2^{\bullet-}$-production by ECPOX (Elstner and Heupel 1976; Rouet *et al.*, 2006). Here, the presence of Mn^{2+} was tested in its activity to modulate ionically bound ECPOX in *P. sativum* seedlings to produce $O_2^{\bullet-}$ via compound III, i.e. just in the presence of H_2O_2, as shown in Fig. 4.1. In the presence of Mn^{2+} a positive correlation between H_2O_2 concentration and $O_2^{\bullet-}$ production was observed. Interestingly, adrenochrome formation was higher in the presence of Mn^{2+} at all concentrations measured (data not shown), but at H_2O_2 concentrations between 100 and 250 µM (*c.* 150 µM),

production of $O_2^{\bullet-}$ was lower than in the absence of Mn^{2+} (Fig. 6.7A). In the absence of Mn^{2+}, maximum rates of $O_2^{\bullet-}$ production were observed at H_2O_2 concentrations of 50 to 100 µM (Fig. 6.7A). Therefore, the mechanisms of $O_2^{\bullet-}$ production in the absence of Mn^{2+} were investigated further. The reaction volume was 1 ml and the B3 fraction was incubated with 50 µM H_2O_2 (50 nmols of H_2O_2). The amount of H_2O_2 was rapidly broken down to 10 ± 0.3 nmols within the first 10 min with no significant ($P < 0.05$) change thereafter, whereas $O_2^{\bullet-}$ production increased for 15 min (Fig. 6.7B). However, the 18.6 ± 0.7 pmols of $O_2^{\bullet-}$ produced during 20 min was over three orders of magnitude lower than the amount of H_2O_2 broken down, showing the vast majority of H_2O_2 was not converted to $O_2^{\bullet-}$ and probably degraded to H_2O and $^3O_2$ (Mika *et al.*, 2004).

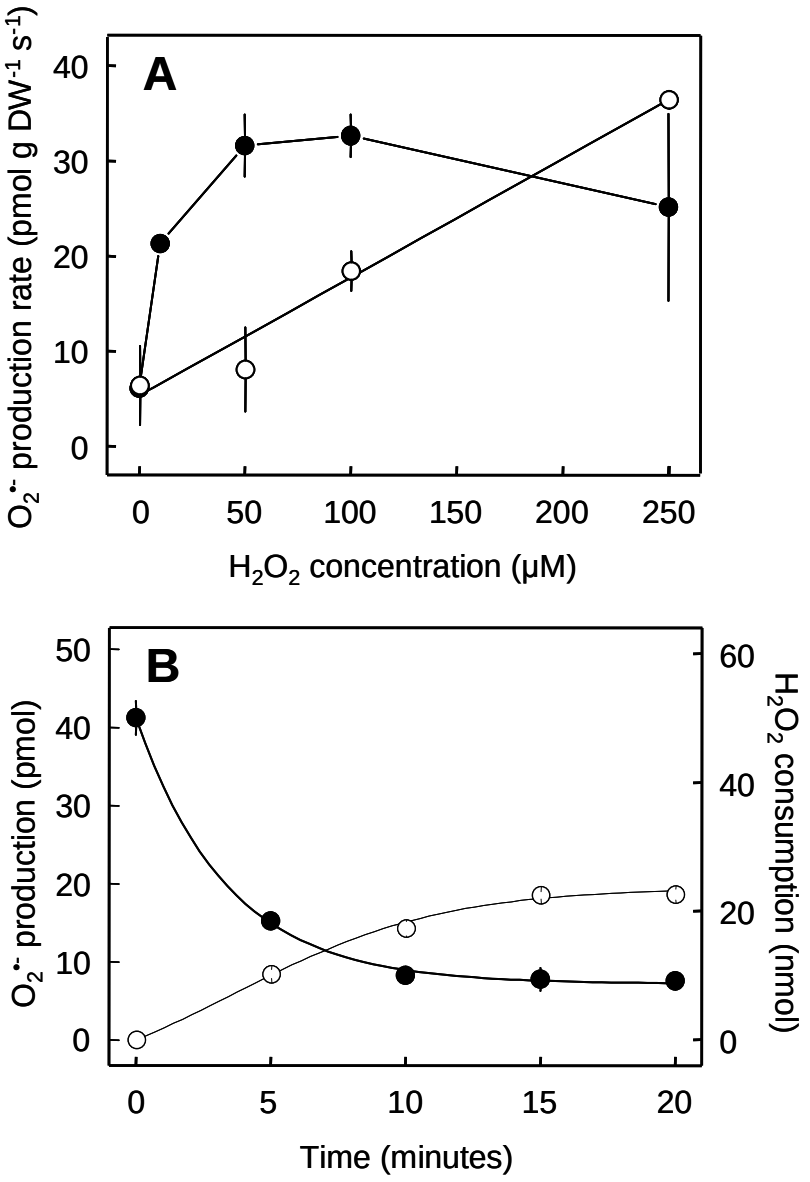


Figure 6.7 Production of $O_2^{\bullet-}$ by ionically bound cell wall enzymes isolated from *Pisum sativum* seedlings using H_2O_2 as an oxidant and reductant. The concentration of ECPOX in the reaction volume (1 ml) was adjusted to 0.2 units ml^{-1} before measurements. **[A]** The B3 fraction was incubated in 1 mM epinephrine, 50 mM K-phosphate buffer at pH 7.0 and various concentrations of H_2O_2 in the presence or absence of 0.5 mM $MnCl_2$, represented by open and closed symbols, respectively. Adrenochrome formation was measured after 12 min of incubation, and $O_2^{\bullet-}$ production was calculated after subtracting non-specific absorbance in the presence of 200 units ml^{-1} SOD (means ± SE, n = 2). **[B]** Breakdown of 50 µM H_2O_2 and production of $O_2^{\bullet-}$ over a 20 min incubation period without $MnCl_2$. Separate incubations were set up for measurements of H_2O_2 (closed symbols, right y-axis) and adrenochrome formation. $O_2^{\bullet-}$ amounts (open symbols, left y-axis) were calculated after subtraction of non-specific absorbance in the presence of 200 units ml^{-1} SOD (means ± SE, n = 4).

Leachates of wounded *P. sativum* seedling axes were collected to investigate released enzymes. To provide an indication of the presence of cytosolic components in the leachate, the activity of glucose-6-phosphate dehydrogenase (G6PDH) was measured (Zhu *et al*., 2006). The activity of G6PDH in the leachate was 0.35 % of total seedling activity, whereas the ECPOX and AOX activity in the leachates were 3.99 % and 4.16 % of the total seedling activity, respectively (n = 4 replicates of 5 axes). Using a combination of PAGE and in-gel activity staining, single bands were observed of 150 kD and 95 kD in size as a result of AOX and LOX activity, respectively (Fig. 6.8). Interestingly, the location of staining for LOX was also a location of staining for ECPOX. ECPOX activity was identifiable in six locations on the gel. These sites included the interface between the stacking and separating gel and five distinct bands between 30 and 95 kD in size. Incubating wounded seedling axes in up to 10 mM NaCl increased the release of ECPOX, but did not lead to a greater release of AOX (Fig. 6.8). This agrees with ECPOX being ionically bound to the cell wall, but that AOX is not (Table 6.1). Interestingly, when leachates were collected in the presence of 5 or 10 mM NaCl, three ECPOX of *c.* 40 to 50 kD in size migrated further within the gels (Fig. 6.8).

In seedling leachates ECPOX activity, quantified in Fig. 6.9A, could be doubled by incubating wounded axes in 5.0 mM NaCl. The activity was halved in measurements taken in the presence of 1 mM NaN_3 (data not shown), suggesting that either ECPOX activity is not entirely inhibited by NaN_3 or other enzymes in leachates unaffected by NaN_3 could oxidise ABTS, as used for quantifying ECPOX activity.

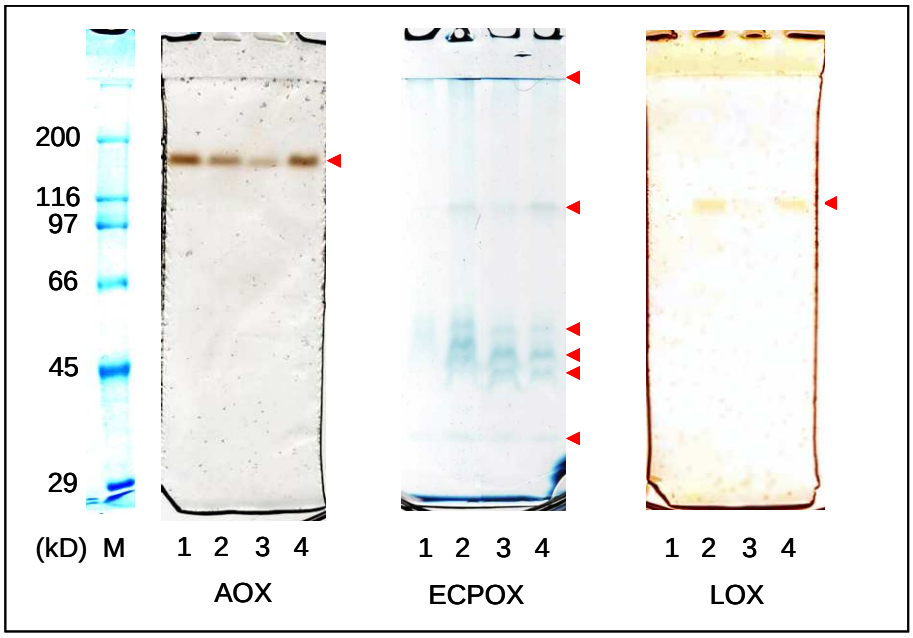


Figure 6.8 Various enzymes in the leachates of wounded *Pisum sativum* seedling axes and a putative role for cyst(e)ine in ECPOX-mediated $O_2^{\bullet -}$ production. From left to right: (M) shows the molecular mass marker in kD; Lanes (1 – 4) of subsequent gels show leachate enzymes collected in the presence of 0, 2.5, 5 or 10 mM NaCl, respectively. AOX were stained using dianisidine in the presence of putrescine and horseradish-POX. ECPOX were stained using TMB in the presence of H_2O_2. LOX were stained using dianisidine in the presence of linoleic acid. Red arrows indicate enzyme activity.

Using HPLC and GC-MS, attempts were made to identify substrates that were leaked from wounded seedling axes and potentially used for the enzymatic production of ROS. Wounding increased the release of glutathione (not shown) and cyst(e)ine (Fig. 6.9B) into the leachate. In *P. sativum* seeds much more glutathione is found relative to cysteine (Kranner and Grill, 1993), whereas in the leachate of wounded seedling axes the amounts of each LMW thiol were insignificantly different (data not shown). Whereas GSH is a well known antioxidant, cyst(e)ine can act as an electron donor to ECPOX (Pichorner *et al.*, 1992; Bolwell *et al.*, 2002) that may support ECPOX-mediated $O_2^{\bullet -}$ production. Wounding increased the oxidation of LMW thiols, e.g. after wounding the percentage of cystine increased from 15 to 26 % of total cyst(e)ine (Fig. 6.9B). The location of ECPOX activity at the interface between the stacking and separating gel also stained for $O_2^{\bullet -}$ production in the presence of H_2O_2 and cyst(e)ine (Fig. 6.9C) (parentheses in cyst(e)ine

highlight the unknown oxidation state given that the incubation also included H_2O_2, which could have oxidised the LMW thiol cysteine to the disulphide cystine). Furthermore, an increase in staining for $O_2^{\bullet -}$ was observed in leachates extracted in the presence of 2.5 mM NaCl, corresponding to the increase in ECPOX staining (Fig. 6.9C). Without the other, cysteine and H_2O_2 were incapable of inducing $O_2^{\bullet -}$ production in-gel. Glutathione, in the presence or absence of H_2O_2, showed no activity (not shown).

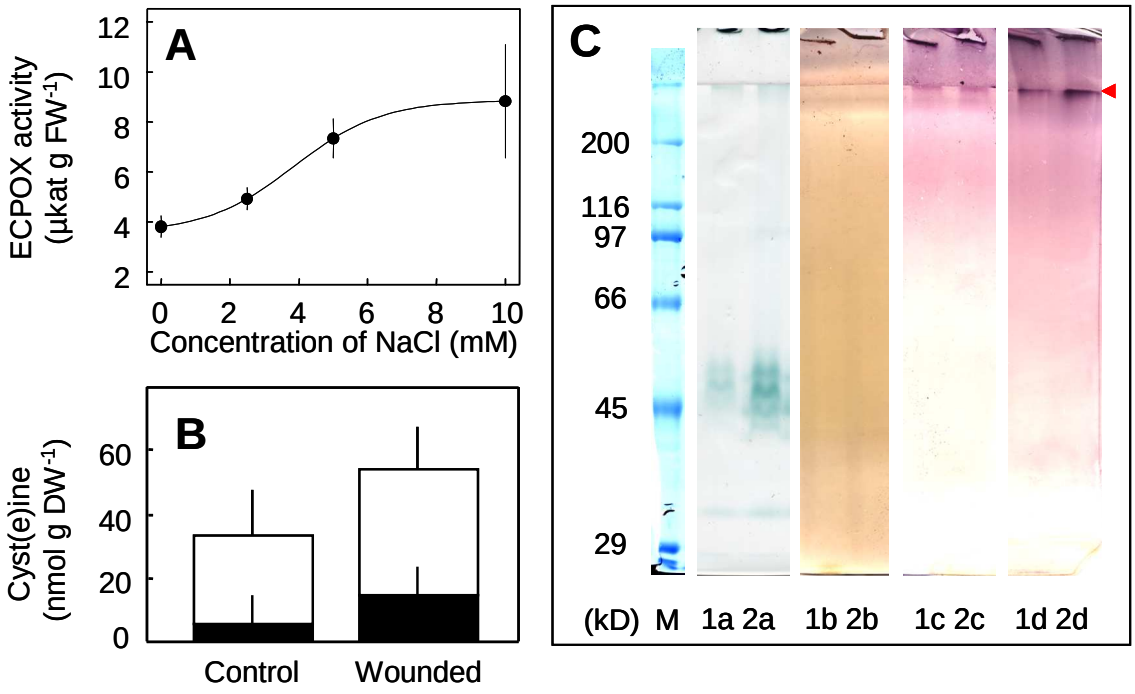


Figure 6.9 Putative role for cyst(e)ine in ECPOX-mediated $O_2^{\bullet -}$ production. **[A]** ECPOX activity, as measured by ABTS oxidation in the presence of exogenous H_2O_2, in leachates extracted in the presence of various NaCl concentrations (means ± SE, 4 replicates of 5 seedling axes). **[B]** Concentrations of cysteine (white bars) and cystine (black bars) in the leachates of non-wounded (control) or wounded seedling axes (n = 4 replicates of 10 seedling axes incubated for 10 min). **[C]** From left to right: (M) shows the molecular mass marker in kD. Lanes (1) and (2) of subsequent gels show leachate enzymes collected in the presence of 0 and 2.5 mM NaCl, respectively. Lanes (a) show ECPOX stained with TMB; In lanes (b – d) $O_2^{\bullet -}$ production is indicated by purple staining in the presence of (b) 10 µM cysteine, (c) 10 mM H_2O_2 or (d) 10 µM cysteine and 10 mM H_2O_2 lanes. Red arrow indicates enzymes producing $O_2^{\bullet -}$.

6.2.3 Identification of substrates that may participate in reactive oxygen species production

Polyamines were detected in leachates (0.5 ± 0.2 nmol g^{-1} DW, n = 10 seedlings during 10 min incubation in 4 ml), but unfortunately due to all peaks in the HPLC chromatograms overlapping, their identities were unresolved. The presence of several fatty acids in the leachates was revealed by GC-MS (Fig. 6.10), including the LOX substrate 9,12-octadecadienoic acid-z,z, (α-linoleic acid), comprising of 0.82 % of total peak area of leachates (Table 6.3).

Table 6.3 GC-MS identification of low-molecular-weight compounds in leachates of wounded *Pisum sativum* seedlings, as shown in Fig. 6.9C. The leachates from wounded seedling axes were composed mainly of sugars, which are not shown. The remaining compounds consisted of polyols and organic acids. Compounds were identified through comparison with the NIST 02 mass spectral library. RSI and SI values are matching factors which describe how well the sample spectra matches that of the library spectra. RSI and SI values above 900 are an excellent match, 800-900 a good match, 700-800 a fair match and below 600 a poor match. The probability is based on the SI match factor and the difference between adjacent matches e.g. if several hits with high SI factors are returned the probability of a correct identification will be low. This often occurs when different isomers have similar mass spectra.

Peak as	Compound	RT (min)	RSI	SI	Probability (%)
1	Palmitelaidic acid	9.98	835	762	82
2	Hexadecanoic acid	10.1	890	869	94
3	9-Tetradecenoic acid	10.63	802	671	20
4	Heptadecanoic acid	10.7	789	625	57
5	9,12-Octadecadienoic acid	11.11	784	712	73
6	trans-9-Octadecenoic acid	11.13	913	875	28
7	Octadecanoic acid	11.28	906	835	89

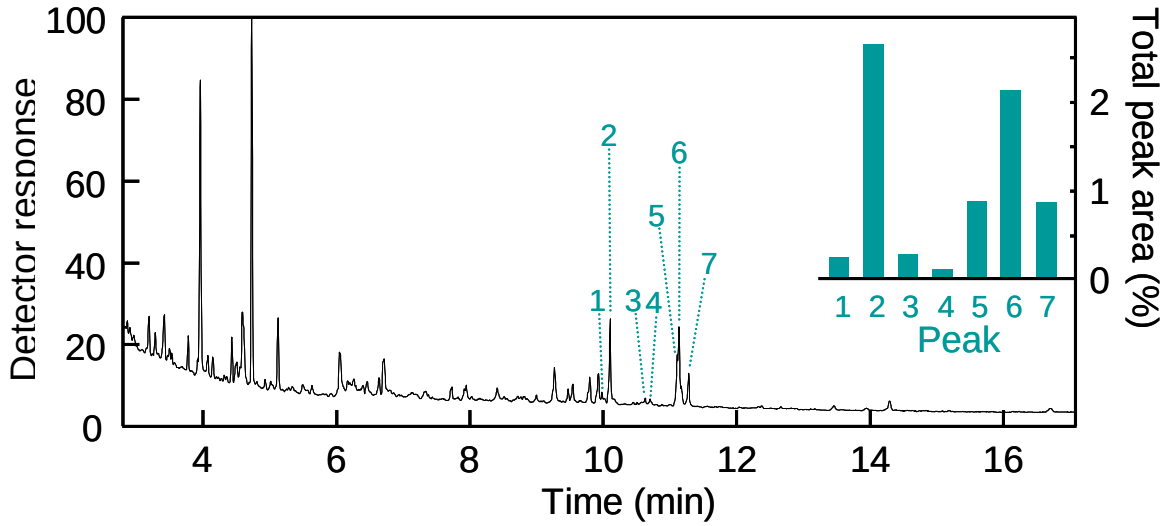


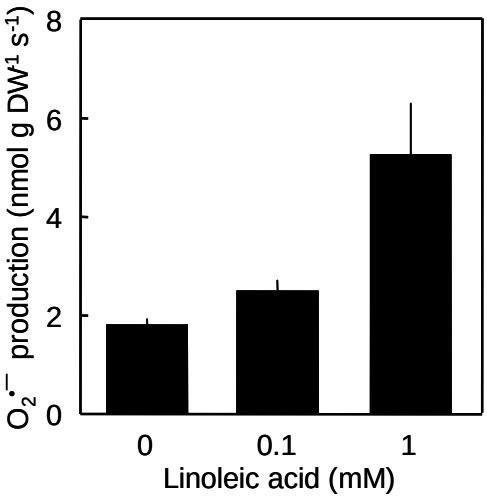
Figure 6.10 Gas chromatogram and relative abundance of compounds in leachates of wounded *Pisum sativum* seedlings, courtesy of Dr Lousie Colville (SCD, RBG Kew, UK). Twenty seedling axes were excised and incubated in 10 ml of H_2O for 5 min. Shown is a typical chromatogram indicating the relative abundance of compounds (left y axis) in the leachate. The peaks labelled 1 – 7 represent the fatty acids shown in Table 6.3. The inset shows the abundance of each compound as a percentage of the total peak area (right y axis) (mean of 2 replicates).

Linoleic acid was further explored in its effect upon the wounding response of seedlings. Exogenously supplying 1 mM of linoleic acid to wounded seedling axes doubled the production of $O_2^{\bullet -}$, whereas carbonic acids and linolenic acid had no affect (Table 6.4). Moreover, the induced $O_2^{\bullet -}$ production in the presence of linoleic acid was concentration dependent (Fig. 6.11).

Table 6.4 Effect of carbonic acids, linoleic, linolenic acid and NADH on $O_2^{\bullet-}$ production by wounded *Pisum sativum* seedlings. The 1.5 ml incubation solution of 50 mM K-phosphate buffer at pH 7.0 contained both epinephrine and compounds at 1.0 mM. The amount of $O_2^{\bullet-}$ produced (nmol g DW^{-1}) after 30 min was calculated as a percentage of measurement in only epinephrine (n = 5 axes per measrurement).

Compound (1.0 mM)	**$O_2^{\bullet-}$ (%)**
2-keto-gluconic acid	104
Galacturonic acid	104
Gluconic acid	099
Linoleic acid	196
Linolenic acid	105
NADH	152

Figure 6.11 The effect of linoleic acid upon $O_2^{\bullet-}$ production by wounded *Pisum sativum* seedlings. The 1.5 ml incubation solution of 50 mM K-phosphate buffer at pH 7.0 contained 1.0 mM epinephrine solution and various concentrations of linoleic acid dissolved in Triton-X. Measurements were after 20 min incubation (means + SE, n = 4 replicates of 5 seedling axes).



6.3 Discussion

6.3.1 Following wounding, AOX are involved in the immediate release of H_2O_2, which is required for $O_2^{\bullet-}$ production

It has been reported that roots, leaves, embryonic axes and fruits respond to wounding by the immediate production of $O_2^{\bullet-}$ (Watanabe and Sakai, 1998; Roach *et al*., 2008; Minibayeva *et al*., 2009) and H_2O_2 (Legendre *et al*., 1993; Gould *et al*., 2002). Following the wounding of *P. sativum* seedling axes, a burst of H_2O_2 was detected with an almost identical temporal pattern reported for punctured leaves of *Pseudowintera colorata* (Gould *et al*., 2002). The fastest rate of H_2O_2 production occurred during the first 5 min (Fig. 6.1A) so that the highest concentration in the incubation solution was observed 8 min after wounding (Fig. 6.1C). Although H_2O_2 production continued from 10 to 20 min at a reduced rate, the actual concentration in the incubation solution fell, probably as a result of H_2O_2 metabolism by catalase and ECPOX as reported earlier (Mika *et al*., 2004). Indeed, the amount of H_2O_2 in the incubation solution containing wounded seedlings increased when both these enzymes were inhibited with NaN_3 (Fig. 6.2A). Moreover, by inhibiting AOX the supply of wounding-induced H_2O_2 became completely depleted to levels observed in the presence of catalase, suggesting AOX are responsible for the all the H_2O_2 production (Fig. 6.2A).

Other studies have shown that wounding-induced H_2O_2 is produced by AOX in chickpea (Rea *et al*., 2002), tobacco (Yoda *et al*., 2006) and maize (Cona *et al*., 2006; Angelini *et al*., 2008). Inhibiting the activity of AOX has critical consequences, such as enhancing the susceptibility of wounded chickpeas to infection (Rea *et al*., 2002). This may be a consequence of reduced lignification and suberin deposition at the wound site (Angelini *et al*., 2008) or losing the capability to initiate cell death (Yoda *et al*., 2006), both due to the retardation of H_2O_2 production. In the study by Cona *et al*. (2006), inhibiting maize AOX lowered H_2O_2 production, but did not affect the release of $O_2^{\bullet-}$ that was strictly dependent upon the activity of NAD(P)H oxidase.

Contrary to this, the immediate release of $O_2^{\bullet-}$ in *P. sativum* seedlings was dependent upon the availability of H_2O_2. Either removing H_2O_2 from the incubation solution with catalase (Fig. 6.1A) or lowering its production by inhibiting AOX (Fig. 6.3C) almost eliminated the immediate wounding-induced production of $O_2^{\bullet-}$. Furthermore, by supplying an exogenous source of H_2O_2 the rate of $O_2^{\bullet-}$ production could be increased (Fig. 6.1D), indicating that the availability of polyamines for AOX to convert to H_2O_2 would affect the initial production of $O_2^{\bullet-}$. AOX (Fig. 6.8) and polyamines were detected in the leachates, but unfortunately more work is required to reveal the identity of the polyamines. Putrescine is the most prevalent polyamine in *P. sativum* seedlings (Flores and Galston, 1982), indicating that it would have been abundant. Supplying an exogenous source of putrescine increased the rate of H_2O_2 production (Fig. 6.3B), suggesting that following wounding, AOX may not have been saturated with substrates. However, considering that measurements were conducted in 5 ml of incubation solution, the naturally occurring polyamines would have been significantly diluted in comparison to their concentration at the wound site. In summary, wound-induced H_2O_2 production by *P. sativum* seedling axes was reliant on apoplastic AOX, which appears to be very important to the production of $O_2^{\bullet-}$ likely to have been mediated by ECPOX.

6.3.2 A role for ECPOX in H_2O_2 metabolism and $O_2^{\bullet-}$ production

Extracellular peroxidases have been identified to be responsible for the production of $O_2^{\bullet-}$ following the wounding of wheat roots (Minibayeva *et al.*, 2001; 2009). Here, inhibiting ECPOX led to a decline in $O_2^{\bullet-}$ production (Fig. 6.2B), where it is assumed that ECPOX are converting H_2O_2 to $O_2^{\bullet-}$ (Halliwell, 1978; Hiner *et al.*, 2001; Kawano, 2003). Between 10 and 20 min after wounding, the contribution of AOX to H_2O_2-derived $O_2^{\bullet-}$ production was finishing (Fig. 6.3C) in accordance with a decline in the rate of H_2O_2 production (Fig. 6.1A). NAD(P)H oxidase may also have provided a small supply of $O_2^{\bullet-}$, which may have dismutated to produce H_2O_2, as inhibiting enzymes that contained flavin active centres significantly ($P < 0.05$) reduced

the amount of $O_2^{\bullet-}$ and H_2O_2 production (Fig. 6.2). However, overall ECPOX had a more significant contribution than NAD(P)H oxidase to wound-induced $O_2^{\bullet-}$ production (Fig. 6.3B).

Epinephrine was used, as in many other studies (Minibayeva *et al.*, 2003; Rouet *et al.*, 2006), to quantify $O_2^{\bullet-}$ production. Some non-specific activity may be expected from spectrophotometric measurements, but adrenochrome formation shown in Figure 6.5A was completely non-specific to $O_2^{\bullet-}$. As epinephrine is a phenolic compound, single electron transfer between H_2O_2 and epinephrine could have been catalysed by ECPOX in the absence of $O_2^{\bullet-}$ (Adak *et al.*, 1998; Chasov and Minibayeva, 2009). Other compounds, such as tetrazolium based compounds XTT (Sutherland and Learmonth, 1997) or NBT (Van-Gestelen *et al.*, 1997) may have been more suitable for $O_2^{\bullet-}$ measurements involving ECPOX. However, XTT is also prone to electron transfer in the absence of $O_2^{\bullet-}$ (Schopfer *et al.*, 2008), further highlighting the difficulties of spectrophotometrically measuring $O_2^{\bullet-}$. The ECPOX activity of B1, B2 and B4 cell wall fractions correlated tightly to adrenochrome formation in the presence of H_2O_2 alone (Fig. 6.5C), suggesting that different ECPOX in the various fractions are capable of oxidising epinephrine. Correlating ECPOX activity to adrenochrome formation also revealed that the B3 fraction may have actually produced $O_2^{\bullet-}$ with H_2O_2 alone, which was explored further.

In chapter 4, it was reported that ionically bound extracellular enzymes of *Castanea sativa* embryonic axes were most efficient in producing $O_2^{\bullet-}$ (Fig. 4.3B). In *P. sativum* seedling axes, a quarter of ECPOX activity was found to be from enzymes ionically bound to the cell wall (Table 6.1). Fielding and Hall (1978) showed that ionically bound enzymes are more prevalent in the cortex of *P. sativum* roots than in the vascular bundle. In the vascular bundle, ECPOX activity is associated to lignification of the xylem (López-Serrano *et al.*, 2004; Tokunaga *et al.*, 2009) whereas the location of the cortex just below the root surface would compliment a role for ionically bound ECPOX in wounding-induced $O_2^{\bullet-}$ production. Ionically bound ECPOX could contribute a

small amount of $O_2^{\bullet-}$ production with H_2O_2 alone (Fig. 6.7A), hypothesised to have occurred via the decay of compound III (Fig. 4.1). However, measurements suggested that only 0.2 % of H_2O_2 was converted to $O_2^{\bullet-}$ (Fig. 6.7B) and this amount is too small to account for the role of ECPOX in wounding-induced $O_2^{\bullet-}$ production (Fig. 6.2B). Therefore, I suggest that ECPOX-mediated $O_2^{\bullet-}$ production, rather than occurring via compound III, requires reductants such as cysteine.

Cysteine has been shown to induce ECPOX to produce H_2O_2 (Bolwell *et al.*, 2002). Pichorner *et al.*, (1992) identified that cysteine was oxidised by horseradish-POX, but H_2O_2 was only produced after all cysteine been oxidised to cystine. Wounding of *P. sativum* seedlings increased the release of cyst(e)ine and also the amount of cystine relative to cysteine (Fig. 6.9B). Using a 2.5 mM salt solution to incubate wounded seedlings increased the release of ECPOX (Fig. 6.9A), which, in the presence of cysteine and H_2O_2, increased the intensity of in-gel staining for $O_2^{\bullet-}$ (Fig. 6.9C). The in-gel activity stain indicated that not all ECPOX were capable of producing $O_2^{\bullet-}$ and both cysteine and H_2O_2 were required in combination for $O_2^{\bullet-}$ production. However, it is reasonable to assume that during the gel staining H_2O_2 would have oxidised LMW thiols to disulphides. Therefore, cystine may be the suitable electron acceptor for ECPOX to produce H_2O_2 that can then be used by ECPOX to produce $O_2^{\bullet-}$ with cysteine as a reductant. This suggests a complex interaction between ECPOX, cysteine, cystine and H_2O_2 in the production of $O_2^{\bullet-}$.

6.3.3 Lipoxygenases may also contribute to the oxidative burst

Similar to ECPOX, LOX uses iron in its active centre to facilitate electron transfer, but unlike ECPOX it is not in a haem group. The in-gel staining of enzymes released by wounded *P. sativum* seedlings suggested either ECPOX or LOX could metabolise linoleic acid or TMB, respectively. The location of a band in the presence of TMB and H_2O_2, a well characterised ECPOX reaction (Thomas *et al.*, 1976), was also the location of band in the presence of

diansidine and linoleic acid (Fig. 6.8). Unless both enzymes are present here, the 95 kD size of the enzyme is more characteristic of a LOX, suggesting that LOX are capable of metabolising TMB in the presence of H_2O_2. This suggests the view of TMB as a specific enzyme ECPOX substrate requires further attention, as does the ability of LOX to metabolise non-fatty acids.

Lipoxygenases can insert a $^3O_2$ molecule into unsaturated fatty acids forming lipid hydroperoxides. In addition to hydroperoxides, LOX have been reported to produce other ROS, such as $^1O_2$ (Kanofsky and Axlerod, 1986) and $O_2^{\bullet-}$ (Zuo *et al.*, 2004). Two dominant LOX isoforms are found in *P. sativum* seeds, LOX-2 and LOX-3 (Mo and Koster, 2006), both identified here after sequencing extracellular enzymes (Table 6.2). LOX-2 can oxidise esterified fatty acids, whereas LOX-3 works primarily with free fatty acids (Hughes *et al.*, 1998). Both isoforms reduced NBT in the presence of NADH (Fig. 6.5D), suggesting either could potentially participate in $O_2^{\bullet-}$ production (Serrano *et al.*, 1994; López-Huertas *et al.*, 1999). Kanofsky and Axelrod (1986) identified that when LOX-2 and LOX-3 are provided with linoleic acid, LOX-3 could convert the hydroperoxides produced by LOX-2 into $^1O_2$. Sequencing proteins indicated that each LOX isoform was present in the sample dominated by the other (Table 6.2), despite the caution taken when isolating individual NBT-stained bands. Thus, it is also possible that LOX-2 and LOX-3 may have operated synergistically to produce $O_2^{\bullet-}$ in the presence of NADH.

LOX are generally associated with intracellular compartments such as the cytoplasm and vacuole (Siedow, 1991; Porta and Rocha-Sosa, 2002), but some have been identified to be attached to the apoplastic side of cell membranes (Sicilia *et al.*, 2005). Furthermore, immunogold labelling with a LOX-antigen has also located the enzyme within the extracellular space of *Phaseolus coccineus* leaves (Skorzynska-Polit *et al.*, 2005), *Hordeum vulgare* root tips (Tamas *et al.*, 2009) and *P. sativum* roots infected with nematodes (Leone *et al.*, 2001), in agreement with an extracellular location of LOX found here (Table 6.2; Fig. 6.6A). Unexpectedly, sequencing the strongest NBT-

stained bands, suggestive of the most efficient $O_2^{\bullet-}$-producing enzymes in the presence of NADH, indicated that dihydrolipoyl dehydrogenases were responsible for the activity (Table 6.2; Fig. 6.6A). These oxidoreductase enzymes are capable of single electron reductions, but are usually located in the mitochondrion (Faure *et al.*, 2000; Starkov *et al.*, 2004). This suggests the extracellular fractions contained some intracellular contamination. However, LOX activity was identified in the leachates derived from wounded *P. sativum* seedlings (Fig. 6.8) that had very low intracellular contamination, suggesting the enzyme was secreted into the apoplast. Furthermore, endogenous linoleic acid enhanced the wounding-induced production of $O_2^{\bullet-}$ (Fig. 6.11).

Linoleic acid is the most abundant fatty acid in phospholipid membranes of non-leaf *P. sativum* cell membranes (Harwood, 1997). The release of membrane-bound fatty acids that is linked to phospholipase activity has been reported to be partially responsible for the induced oxidative burst of soybean and tobacco cells (Chandra *et al.*, 2000; Mathieu *et al.*, 2002). Moreover, H_2O_2 has been shown to enhance LOX activity of soybean seedlings (Marcí *et al.*, 1994), including the induction of $^1O_2$ production (Kanofsky and Axlerod, 1986), which could also explain the effect of H_2O_2 on enhancing wound-induced $O_2^{\bullet-}$ production (Fig. 6.1D). Thus, it is plausible that LOX are associated with the wounding-induced oxidative burst beyond their established contribution to other wounding associated defences. The presence and metabolism of different fatty acids are associated with unique stress responses. For example, linoleic acid stimulated the wounding-induced production of $O_2^{\bullet-}$, whereas linolenic acid did not (Table 6.4), but systemic wound defences are reliant upon jasmonates that are putatively derived from LOX metabolism of linolenic acid (Léon *et al.*, 2001; Wasternack, 2006; Yang *et al.*, 2009). Other LOX-3 reaction products include carbonyl compounds (Hughes *et al.*, 1998), such as malonaldehyde (MDA), which accumulates at wound sites (Vollenweider et al., 2000). Carbonyls, such as MDA, up-regulate stress-related genes (Weber *et al.*, 2004), as well as initiate other beneficial responses to wounding (Porta and Rocha-Sosa, 2002).

6.3.4 Summary

The aim set out at the beginning of this chapter was to clarify the mechanisms of ROS during the wounding-induced oxidative burst of *P. sativum* seedling axes. Characterising the pattern of ROS production, elucidating enzyme participation and combining cell wall fractionation, in-gel activity staining and protein sequencing identified several enzymes that contribute to the production of H_2O_2 and $O_2^{\bullet-}$. The substrates potentially used by ROS-producing enzymes were also investigated in the leachates of wounded seedling axes using HPLC and GC-MS. Upon wounding, AOX immediately produced H_2O_2, that was essential for ECPOX-mediated $O_2^{\bullet-}$ production in agreement with the original hypothesis. Wounding induced the release of cyst(e)ine, which in the presence of H_2O_2, was capable of inducing ECPOX from the leachates to produce $O_2^{\bullet-}$. Surprisingly, LOX were identified in the ionically bound cell wall fraction and also in the leachates of wounded seedlings. Linoleic acid, also released by wounded seedlings, enhanced the production of $O_2^{\bullet-}$ when added exogenously. Overall, this suggests that LOX, with AOX and ECPOX, also contributed to the wounding-induced oxidative burst. In contrast to other studies, inhibitor evidence suggested only a small contribution to H_2O_2 and $O_2^{\bullet-}$ production by NAD(P)H oxidase. Further tests with gene knock-out or antisense mutants may reveal the interaction of the numerous enzymes identified here that all contribute to the wounding induced oxidative burst.

Chapter 7

Conclusions

The production of ROS, depending upon intensity and sub-cellular localization can cause oxidative stress. As a marker of oxidative stress during desiccation, a major stress for recalcitrant seeds, the redox potentials of LMW redox couples were measured in *C. sativa* seeds. The increased oxidation of LMW thiols and accumulation of their corresponding disulphides during desiccation was considered indicative of disrupted metabolism leading to enhanced ROS production that had 'negative' effects of ROS and caused oxidative stress. Glutathione was the most abundant LMW thiol and thus, the most influential of all LMW thiols on the cellular redox homeostasis. Non-desiccated embryonic axes contained double the amount of total glutathione than the cotyledons, suggesting that embryonic axes have a better constitutive protection from oxidative stress. In accord with previous findings that embryonic axes respire more than cotyledons (Leprince *et al*., 1999), axes appeared to be more prone to oxidative stress as indicated by greater accumulation of LMW disulphides, which led to a less negative $E_{RSSR/2RSH}$ compared to the cotyledons. However, the recovery of embryonic axes from non-germinating seeds using embryo rescue techniques reflected the greater stress tolerance of isolated axes over the whole seed. Measuring the activity of ROS-processing enzymes and other antioxidants during desiccation, such as glutathione reductase, would have provided a more conclusive analysis and would be an interesting focus for future research, as it is still not fully understood whether or not desiccation tolerance of orthodox seeds and the lack thereof in recalcitrant seeds correlate with the success or failure, respectively, of the antioxidant machinery.

In agreement with changes in $E_{GSSG/2GSH}$, a recent study has shown that desiccating recalcitrant seeds increases the production of ROS (Cheng and Song, 2008). In that study, seeds were homogenised to provide an overall measurement of ROS. Therefore, as in most other previous studies on the

production of ROS in recalcitrant seeds, intra- and extracellularly produced ROS could not be distinguished. However, such a distinction is the basis to provide an understanding of both the 'negative' and the 'positive' effects of ROS. This thesis shows that during desiccation of *C. sativa* embryonic axes, the pattern of extracellular $O_2^{\bullet-}$ production was distinctly different from the intracellular redox environment. Initially, desiccation led to a steep increase in the production of extracellular $O_2^{\bullet-}$, which peaked in stressed embryonic axes when most had retained viablity and declined with major viability losses, while significant changes in $E_{GSSG/2GSH}$ were observed later in the time course of desiccation. The bell-shaped production of extracellular $O_2^{\bullet-}$during desiccation could be deemed typical of a stress response.

To investigate further the production of $O_2^{\bullet-}$ by desiccation intolerant tissue, the loss of desiccation tolerance during germination of orthodox *P. sativum* seeds was used as a model. Measurements of extracellular $O_2^{\bullet-}$ production following the desiccation of embryonic axes and seedling axes of *P. sativum* confirmed that $O_2^{\bullet-}$ production was linked to desiccation intolerance. In *P. sativum* seedling axes, the induced production of extracellular $O_2^{\bullet-}$ was particularly intense in the region of the radicle that remained viable and the site of secondary root growth. Therefore, extracellular ROS may play important 'positive' roles in stress response. However, neither the presence of, nor the mechanisms by which, ROS are produced by seeds in response to the stresses that may accompany germination and viability loss have been characterised.

A 'positive' side to ROS is their involvement in signal transduction including a specific role for $O_2^{\bullet-}$ in root and root-hair growth (Foreman *et al.*, 2003). The ability of ROS to reversible 'switch' redox sensitive proteins combined with a precise control over their production and removal provides ROS with their valuable signalling capabilities (Rhee, 2006). Treating desiccation-stressed *C. sativa* seeds and embryonic axes with H_2O_2 could prevent desiccation induced viability loss during germination and enhance vigour, respectively. Furthermore, *P. sativum* seedling axes, at the stage of

first root hair growth, had improved establishment of secondary roots when treated with H_2O_2 after their radicle meristem had been lost to desiccation. Interestingly, H_2O_2 treatments of *C. sativa* embryonic axes and *P. sativum* seedling axes affected root growth more than shoot formation, suggesting differences in the influence of ROS on organ development above and below ground. However, antioxidants such as ascorbate, are also associated with cell proliferation (Arrigoni and De Tullio, 2002), requiring that concentrations of ROS are very carefully regulated and localised to avoid their 'negative' effects.

To add complexity to the activities of ROS and antioxidants, some antioxidants can also participate in the production of ROS, which has been given consideration in this thesis. Ferulic acid and cysteine have been associated with antioxidant activity, but can act as pro-oxidants in the presence of transition metals (Sakihama *et al*., 2002), after becoming oxidised (Pichorner *et al*., 1992; Takahama, 2004) or in the presence of H_2O_2 and ECPOX (Kukavica *et al*., 2009). Wounding of *P. sativum* seedling axes induced the release of cyst(e)ine, which in the presence of H_2O_2, induced ECPOX in the leachates to produce $O_2^{\bullet-}$. Relative to GSH, the apoplastic amounts of cyst(e)ine were much greater than the amounts found in the seedling axis. As seedling axes contained less cystine compared to cysteine, cystine may have been selectively leaked into the apoplast where it may induce ECPOX to produce $O_2^{\bullet-}$. Ferulic acid was another compound that was able to induce the production of $O_2^{\bullet-}$ from cell wall enzymes. Future research into unravelling when and how this switch in activity is regulated may lead to the discovery of the elusive ECPOX electron donor used in producing ROS.

Extracellular ROS were produced when *C. sativa* embryonic axes or *P. sativum* seedling axes were wounded. Even after only 6 h of imbibing *P. sativum* seeds, excising the embryonic axis from the cotyledon induced the production of $O_2^{\bullet-}$. The oxidative burst following stress is a typical plant response, but to the best of my knowledge, this is the first report of a wounding-induced oxidative burst in seeds. An ECPOX capable of producing

ROS in the presence of a range of electron donors was identified in 3 h imbibed *P. sativum* seeds, suggesting that $O_2^{\bullet-}$-producing ECPOX are synthesised during seed maturation. In the desiccated state, cell membranes of orthodox seeds are protected from conformational changes (Hoekstra *et al.*, 1997), which could hamper the activity of trans plasma-membrane proteins, such as NAD(P)H oxidases in producing $O_2^{\bullet-}$. Accordingly, in *P. sativum* seedling axes inhibiting ECPOX had a more significant effect in lowering the wounding-induced production of $O_2^{\bullet-}$ than inhibiting NAD(P)H oxidases. In contrast to orthodox seeds, the plasma membranes in recalcitrant embryonic axes are intact from maturation to germination. Incubating *C. sativa* embryonic axes with inhibitors indicated that NAD(P)H oxidases and ECPOX could have been equally involved in producing $O_2^{\bullet-}$, although the specificity of DPI to inhibiting NAD(P)H oxidases was questioned.

Cell walls of *C. sativa* and *P. sativum* embryonic axes and seedling axes, respectively, were fractionated to identify extracellular enzymes capable of producing ROS. In the cell wall fractions of both species, ionically bound cell wall enzymes were identified to be most active to produce $O_2^{\bullet-}$. However, measurements of ROS in the presence of enzyme inhibitors suggested the contribution of several ROS-producing enzymes that may integrate together to become functional. For example, results in chapter 6 showed that wounding-induced H_2O_2 production by AOX was essential for ECPOX to produce $O_2^{\bullet-}$. Most AOX were loosely associated to the cell wall and could be leaked into the incubation solution following wounding. Several ECPOX were also identified in the leachates of wounded *P. sativum* seedling axes, in agreement with other reports that have shown that ECPOX are released in response to stresses (Sgherri *et al.*, 2007; Minibayeva *et al.*, 2009). The release of four ECPOX could be increased by incubating wounded axes with 2.5 mM NaCl. An increase in the apoplastic ion concentration may regulate the release of ECPOX in response to stress, which could also be critical for the enzyme's activity to produce $O_2^{\bullet-}$. Furthermore, the results also suggested that apoplastic LOX contribute to the wounding-induced production of $O_2^{\bullet-}$.

Thus, AOX, ECPOX and LOX could all be essential in the oxidative burst in response to wounding and other abiotic stresses, such as desiccation.

Studies in this thesis are among the first reports to show that extracellular ROS are produced by embryonic axes or seedling axes and the first to report on the putative production mechanisms by cell wall enzymes, implicating ECPOX and LOX in $O_2^{\bullet -}$ production. Moreover, the results suggest that in certain time windows, ROS participate in signalling pathways involved in stress response, regeneration and growth.

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