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Extracellular production of reactive oxygen species during seed germination and early seedling growth in *Pisum sativum*

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ABSTRACT

Extracellularly produced reactive oxygen species (ROS) play key roles in plant development, but their significance for seed germination and seedling establishment is poorly understood. Here we report on the characteristics of extracellular ROS production during seed germination and early seedling development in *Pisum sativum*. Extracellular superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) production and the activity of extracellular peroxidases (ECPOX) were determined spectrophotometrically, and $O_2^{\bullet -}$ was identified by electron paramagnetic resonance. Cell wall fractionation of cotyledons, seed coats and radicles was used in conjunction with polyacrylamide gel electrophoresis to investigate substrate specificity and molecular masses of $O_2^{\bullet -}$-producing enzymes, and the forces that bind them to the cell wall. Seed imbibition was accompanied by an immediate, transient burst of redox activity that involved $O_2^{\bullet -}$ and other substances capable of oxidizing epinephrine, and also H_2O_2. At the final stages of germination, coinciding with radicle elongation, a second increase in $O_2^{\bullet -}$ but not H_2O_2 production occurred and was correlated with an increase in extracellular ECPOX activity. Electrophoretic analyses of cell wall fractions demonstrated the presence of enzymes capable of $O_2^{\bullet -}$ production. The significance of extracellular ROS production during seed germination and early seedling development, and also during seed aging, is discussed.

Introduction

Reactive oxygen species (ROS), including the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) are metabolic by-products in by both plants and animals. These ROS can directly attack proteins, lipids and nucleic acids if not sufficiently controlled by antioxidants (Halliwell and Gutteridge, 2006). However, despite these harmful effects, ROS also act as secondary messengers in signal transduction pathways that control processes as diverse as plant growth and development, stress response and programmed cell death (Gechev et al., 2006). In particular, extracellularly produced ROS are important components of plant disease resistance. Following wounding or pathogen attack, many plants produce a rapid, transient oxidative burst of ROS that can be directly toxic to pathogens (Mika et al., 2004). In addition, they play a vital role in growth by facilitating the cell wall loosening required for cell elongation (Passardi et al., 2004, 2006). In seeds, intracellular ROS formation has been frequently investigated (Bailly, 2004, for review; Wojtyla et al., 2006). Although several studies have shown extracellular ROS production during seed germination (Puntarulo et al., 1988; Schopfer et al., 2001), surprisingly little information is available on the physiological significance of extracellular ROS production for seed germination and early seedling development.

Recently, we demonstrated that the embryonic axes of recalcitrant (desiccation sensitive) sweet chestnut (*Castanea sativa*) seeds produced a burst of $O_2^{\bullet -}$ in response to wounding that was modulated by desiccation (Roach et al., 2008). Several enzymatic mechanisms for extracellular ROS formation in plants during stress and development have been proposed, involving extracellular peroxidases (ECPOX) bound to cell walls (Bindschedler et al., 2006), plasma membrane NAD(P)H oxidases (Sagi and Fluhr, 2006), amine oxidases (Cona et al., 2006) and oxalate oxidases (Bernier and Berna, 2001). Moreover, lipoxygenases (LOX) can generate $O_2^{\bullet -}$ via oxidation of pyridine nucleotides (Roy et al., 1994). Our later work on sweet chestnut seeds indicated that, in this species ECPOX were responsible for extracellular $O_2^{\bullet -}$ formation (Roach et al., 2010).

The first aim of the present investigation was to determine the patterns of extracellular $O_2^{\bullet -}$ and H_2O_2 production during seed germination and early seedling development in garden pea (*Pisum sativum*), and to determine how artificial seed aging affects

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these patterns. Pea seeds are orthodox (desiccation tolerant), allowing us to compare results with our earlier work on sweet chestnuts seeds. Secondly, we investigated the mechanisms responsible for extracellular ROS formation. For this purpose, we tested the sensitivity of $O_2^{\bullet -}$ production to peroxidase (POX) and NADPH oxidase inhibitors. We also measured changes in ECPOX activity during seed germination. Additionally, we used a cell wall fractionation technique in combination with polyacrylamide gel electrophoresis (PAGE) to determine the forces that bind the $O_2^{\bullet -}$-producing enzymes to the cell walls. The overall aim of the work presented here was to characterize the patterns of ROS production in relation to radicle protrusion in a model orthodox seed, with the broader aim to increase our understanding of the roles of extracellular ROS production in seeds.

Materials and methods

Plant material and chemicals

Organically grown seeds of Garden pea (*Pisum sativum* L. cv. Rondo) were obtained from Kings Seeds (Colchester, Essex, UK) and stored at 15 °C and 15% relative humidity (RH) until use. Analytical grade chemicals were purchased from Sigma, (St. Louis, MO, USA), Fisher (Loughborough, Leicestershire, UK) and Fluka (Buchs, Switzerland) and all solutions were made with distilled deionized water unless indicated otherwise. "Broad Range" molecular mass markers were obtained from Bio-Rad (Hercules, CA, USA).

Germination testing and seed moisture content

Seeds were germinated on moist filter paper at 25 °C and an 8 h light (15 μmol m^{-2} s^{-1} warm fluorescent light)/16 h dark cycle ($n=5$ replicates of 20 seeds). Seed viability was assessed by the percentage of germinated seeds (total germination; TG). Germination was defined as radicle elongation after testa rupture by at least 2 mm (for whole seeds) or as radicle elongation to at least 6 mm (for de-coated seeds). Seed moisture content (MC) was determined after heating at 103 °C for 17 h and expressed on a fresh weight (FW) basis.

Aging and de-coating treatments

To accelerate aging, seed MC was increased by equilibrating seeds with an initial MC of 6.6 ± 0.1% for 5 weeks in tightly sealed boxes at 20 °C over 29% (w/v) LiCl solution (62% RH, recorded with a Rotronic AWVC-D10 Hygropalm) until their MC was stable at 11.7 ± 0.1%. Equilibrated seeds were then aged at 45 °C over 25% (w/v) LiCl solution, generating 71% RH. These equilibrated seeds were used for further experimentation after 0 d (non-aged controls; TG = 98 ± 1%), 29 d (TG = 50 ± 0%) and 102 d (TG = 0 ± 0%); $n=5$ replicates of 20 seeds each. To evaluate the contribution of seed parts to overall $O_2^{\bullet -}$ production, seed coats were removed from embryos ($n=5$ replicates of 10 seeds) after soaking in distilled water for 3 h (MC = 35 ± 3%), both dried in a desiccator above silica gel (*c.* 3% RH) at 20 °C to their initial MC, and then stored in airtight foil bags at 5 °C until experimentation.

Extracellular ROS production and ECPOX activity during seed germination and early seedling development

For ROS measurements, 5 replicates of 10 seeds each were tested at various time intervals until germination was complete, using the same seeds during germination; between measurements seeds were kept imbibed on wet filter paper. In later experiments (insets in Fig. 1), the same seeds were used only once, and germinated seeds grouped according to radicle length and $O_2^{\bullet -}$ production rates measured. Seeds were treated the same way for determination of ECPOX activity, except that the same seeds were used only for one measurement.

Extracellular $O_2^{\bullet -}$ production was estimated after Minibayeva et al. (2009) by shaking seeds or young seedlings at 45 rpm in 15 mL of 1 mM epinephrine, pH 7.0, for 30 min at 25 °C and the $O_2^{\bullet -}$-dependent oxidation of epinephrine to adrenochrome was followed spectrophotometrically (A_{490}; $\varepsilon = 4.02$ mM^{-1} cm^{-1}). In a second assay, plant material was incubated in 15 mL 0.5 mM sodium 3′[-1-(phenylamino-carbonyl-carbon)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulphonic acid hydrate (XTT) and 0.2 mM NADH for 30 min at 25 °C at pH 7.0 (A_{470}; $\varepsilon = 21.6$ mM^{-1} cm^{-1}). Spontaneous oxidation of epinephrine and XTT were minimal during the time course of the assay. To check for

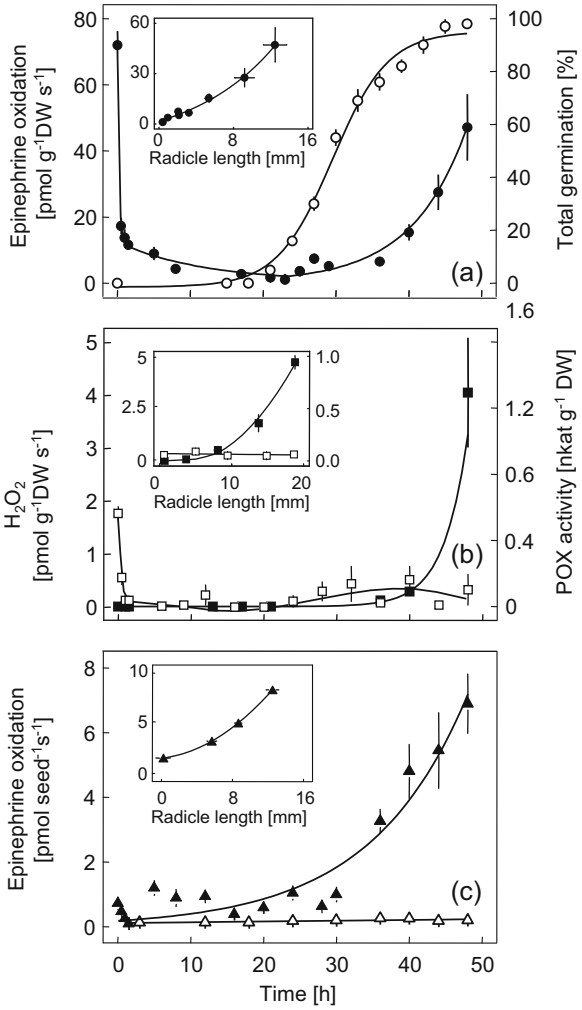

Fig. 1. Rates of extracellular $O_2^{\bullet -}$, assessed as epinephrine oxidation, and H_2O_2 production, and activity of released ECPOX during seed germination and early seedling development in *Pisum sativum*. Epinephrine oxidation during imbibition could not be exclusively attributed to $O_2^{\bullet -}$ (see text for details). (a) TG (open circles) and extracellular $O_2^{\bullet -}$ production (closed circles). Inset, correlation between radicle length and $O_2^{\bullet -}$ production ($R^2=0.99$, $P<0.0001$). (b) H_2O_2 production (open squares) and ECPOX activity (closed squares). Inset, relationship between radicle length and ECPOX activity (solid squares; $R^2=0.99$, $P<0.007$) and H_2O_2 production (open squares; no correlation). (c) Contribution of seed coats (open triangles) and embryos (solid triangles) to extracellular $O_2^{\bullet -}$ production shown on a per-seed basis. Inset, correlation ($R^2=0.99$, $P<0.003$) between $O_2^{\bullet -}$ production and radicle length. Values are given as ± SE, $n=5$.

Table 1
Effect of SOD on $O_2^{\bullet -}$ production by leachates derived during the initial oxidative burst of *Pisum sativum* seeds imbibed for 30 min and from seedlings 48 h after the onset of imbibition. SOD was added to the leachates together with the epinephrine or XTT and $O_2^{\bullet -}$ production was detected after 30 min. Values are given ± standard error, $n=5$. Within each column for each $O_2^{\bullet -}$ trap, values followed by the same letter do not differ significantly ($P<0.05$).

	$O_2^{\bullet -}$ production (pmol g^{-1} DW s^{-1})	
Reagents added	Initial oxidative burst	Second increase in $O_2^{\bullet -}$ production (48 h)
Epinephrine (1 mM)	27.8 ± 4.2 (100%)[a]	13.9 ± 2.8 (100%)[a]
Epinephrine+SOD (250 units mL^{-1})	19.4 ± 1.9 (70%)[a]	2.8 ± 0.6 (20%)[b]
XTT (0.5 mM)+NADH (0.2 mM)	0 ± 0[a]	0.44 ± 0.06 (100%)[a]
XTT+NADH+SOD (250 units mL^{-1})	0 ± 0[a]	0.01 ± 0.00 (2.3%)[b]

specificity, $O_2^{\bullet -}$ production was measured in the presence of 250 units mL^{-1} superoxide dismutase (SOD). Assays were also carried out with 250 units mL^{-1} SOD using leachates rather than plant material (the cell wall can limit access of SOD to $O_2^{\bullet -}$ producing sites). Rates of $O_2^{\bullet -}$ production were lower in leachates in the absence rather than the presence of plant tissue (Table 1, Fig. 1). The presence of $O_2^{\bullet -}$ was confirmed by electron paramagnetic resonance (EPR, radiospectrometer RE 1306, Smolensk, Russian Federation) with the spin trap Tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid disodium salt) as described by Roach et al. (2008). Whole seedlings were also stained with nitroblue tetrazolium (NBT) to visualize $O_2^{\bullet -}$ production at the tissue level. Extracellular H_2O_2 production was measured using the xylenol orange assay after shaking material in 15 mL of distilled water, pH 7.0 for 30 min (Minibayeva et al., 2009).

ECPOX activity of whole seeds and POX activity in cell fractions (see below) were investigated spectrophotometrically (Childs and Bardsley, 1975) 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 ($ABTS^+$) at A_{420} ($\varepsilon=36\ mM^{-1}\ cm^{-1}$). For whole seeds measurements, seeds were incubated in 5 mL of 1 mM ABTS dissolved in 50 mM phosphate buffer, pH 6.0 containing 10 mM H_2O_2, and the absorption of the incubation solution was recorded for 10–15 min at 25 °C. For cell wall fractions, the assay mixture (1 mL) contained 10–200 μl of extract, 1 mM of ABTS in 50 mM phosphate buffer, pH 6.0 and 10 mM H_2O_2.

Inhibition of putative $O_2^{\bullet -}$-producing enzymes

The effects of the following enzyme inhibitors on epinephrine oxidation in seed leachates were tested: 1 mM KCN, 1 mM sodium azide (NaN_3), and 10 μM diphenylene iodonium chloride (DPI) in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 1%, and DMSO was added at the same concentration to all other solutions in this experiment. Inhibitor solutions (pH 7.0) were added concurrently with epinephrine and $O_2^{\bullet -}$ production rates estimated over 30 min. To test whether the initial oxidative burst is an enzyme-catalyzed event, $O_2^{\bullet -}$ production was measured after imbibing seeds at a range of temperatures.

Cell wall fractionation of radicles, cotyledons and seed coats

Radicles, cotyledons and seed coats were separated 48 h after the onset of imbibition, immediately frozen in liquid nitrogen, freeze-dried, and fractionated after Rast et al. (2003), yielding a crude cell fraction (C), enzymes loosely bound to the cell wall (B1), and enzymes bound to the cell wall by hydrophobic interactions, ionic and covalent bonds (B2, B3 and B4, respectively).

Electrophoresis

Cell wall fractions were dialyzed against solid sucrose, followed by reverse dialysis overnight at 4 °C against 50 mM phosphate buffer pH 7. The sample was then microconcentrated using "Microcon" centrifugal filters (10 000 MW cut off) (Millipore, Billerica, MA, USA). Samples were aliquoted and used immediately, or stored at −70 °C. Electrophoretic studies were carried out using a modification of the method of Laemmli (1970) with 12% polyacrylamide gels. Running buffer and gels contained 0.1% SDS, but samples were not heated, and mercaptoethanol and SDS were omitted from the loading buffer. "Broad Range" molecular mass markers, stained by Coomassie brilliant blue G250, were used for assessing the relative molecular masses of proteins.

After electrophoresis, ECPOX activity was visualized by in-gel staining in 0.25 M sodium acetate buffer, pH 5.0, containing 10% glycerol, using 20 mM guaiacol and 20 mM H_2O_2. Haem groups were stained with 6.3 mM 3,3′,5,5′-tetramethyl-benzidine (TMB) in 0.25 M sodium acetate buffer, pH 5.0, and 30 mM H_2O_2 (Thomas et al., 1976). Lipoxygenase activity was visualized by in-gel staining using 20 mM linoleic or linolenic acid in 0.1 M phosphate buffer, pH 6.0 (Manchenko, 2002). Superoxide production was shown after staining with 0.5 mM NBT and either 0.4 mM NADH or 0.2 mM NADPH (López-Huertas et al., 1999). Incubating gels in the absence of NADH or in the presence of 100 units mL^{-1} SOD completely prevented di-formazan formation.

Statistical analysis

Data were analyzed for significance by one- or two-way ANOVA in combination with the LSD test for post-hoc comparisons of means. Arcsine transformation was applied to TG and axis viability data to simulate a normal distribution of data.

Results

Redox activity during germination

The initial stages of seed imbibition were accompanied by a transient burst of extracellular H_2O_2 production and epinephrine oxidation (Fig. 1a, b). After 25 h, a steady increase in epinephrine oxidation occurred, coinciding with radicle emergence, while the seeds produced only trace amounts of H_2O_2. Germination was completed after 48 h (TG = 98%). The specificity of the epinephrine assay for $O_2^{\bullet -}$ was tested by examining the effects of SOD on leachates derived from seeds, by using an alternative assay with XTT, and by using EPR. SOD inhibited the initial transient increase in epinephrine oxidation by only 30%, suggesting that agents

other than $O_2^{\bullet -}$ were oxidizing the epinephrine (Table 1). By contrast, the second increase in epinephrine oxidation was inhibited by 80% by SOD (Table 1). Using XTT, $O_2^{\bullet -}$ production was not detectable during the initial burst of epinephrine oxidation in leachates derived from freshly imbibed seeds, but leachates derived later during germination could readily reduce XTT, and the reduction was highly SOD sensitive (Table 1). EPR confirmed that, while only small amounts of $O_2^{\bullet -}$ were produced during imbibition, post-germination ROS production correlated with strongly enhanced $O_2^{\bullet -}$ production (Fig. 2a). In seeds repeatedly imbibed and dried, the burst of epinephrine oxidation only occurred during the first imbibition. After four cycles of imbibition and drying, seeds were allowed to germinate, and the second increase in ROS production occurred in the same way as in untreated seeds (data not shown).

Seed parts responsible for ROS production

In germinating seeds, $O_2^{\bullet -}$ production was strongly correlated with radicle length (inset of Fig. 1a). The seed coat only produced $O_2^{\bullet -}$ at low rates (Fig. 1c), suggesting that the embryos were responsible for most of the $O_2^{\bullet -}$ production in germinating seeds. The strong correlation between radicle length and $O_2^{\bullet -}$ production in de-coated embryos (Fig. 1c, inset) clearly suggested that radicles produced the majority of the $O_2^{\bullet -}$. Staining with NBT showed that $O_2^{\bullet -}$ was initially produced at the elongation zone of the roots of young seedlings with root lengths under 2 cm. In older seedlings with root lengths greater than 4 cm, $O_2^{\bullet -}$ production occurred patchily across the whole root surface and at the root cap (Fig. 2b). Imbibition of de-coated and re-dried seeds or seed coats was not accompanied by epinephrine oxidation, but $O_2^{\bullet -}$ production by embryos later during germination increased in the same way as in seeds with intact coats (Fig. 1c).

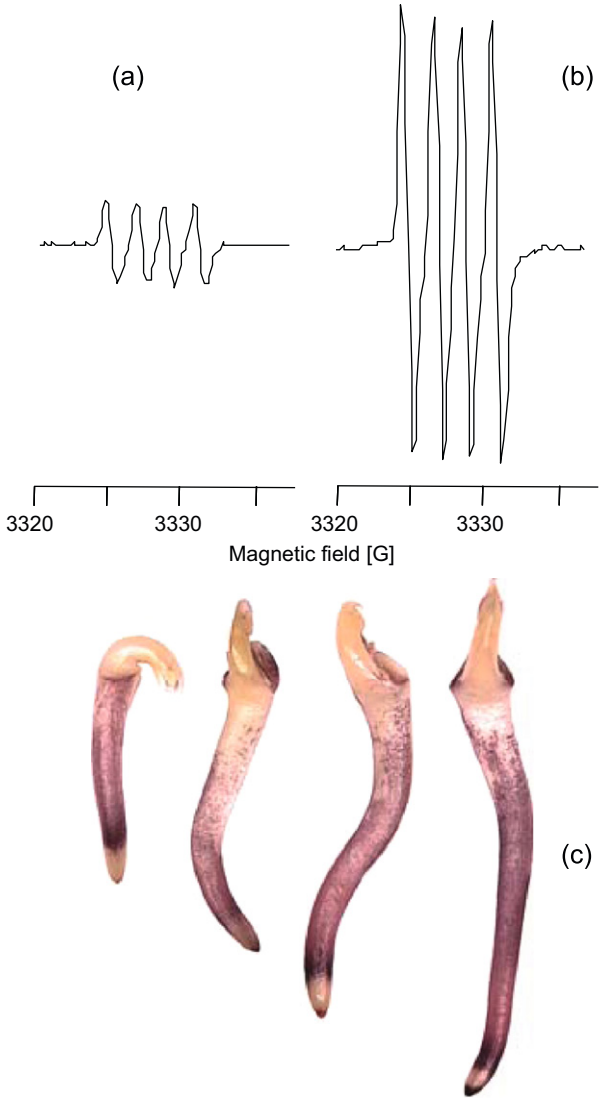

Fig. 2. Typical ESR spectra of the Tiron semiquinone radical formed as a result of $O_2^{\bullet -}$ production in leachates derived (a) during the initial oxidative burst of *Pisum sativum* seeds imbibed for 30 min and (b) of seedlings 48 h after the onset of imbibition. (c) Visualization of $O_2^{\bullet -}$ production after staining of seedling axes with NBT. Dark purple staining indicates $O_2^{\bullet -}$ production.

Effects of seed aging on ROS production

Aging of *P. sativum* with their MC raised to 11.7% in air of 11.7% RH, progressively decreased their ability to germinate; after aging for 29 and 102 d, TG declined to 50% and 0%, respectively (Fig. 3). The seed lot with 50% germination showed the same overall, but delayed pattern of epinephrine oxidation, with approximately halved rates as compared to non-aged controls, indicating that individual viable seeds produced $O_2^{\bullet -}$ at similar rates.

Seeds with TG = 0 produced a small initial burst of epinephrine oxidation, but none later.

Identity of ROS producing enzymes

Sodium azide and DPI had no effect on the initial epinephrine oxidation (not shown), but tended to reduce $O_2^{\bullet -}$ production later during germination, and KCN reduced it by over 90% (Table 2).

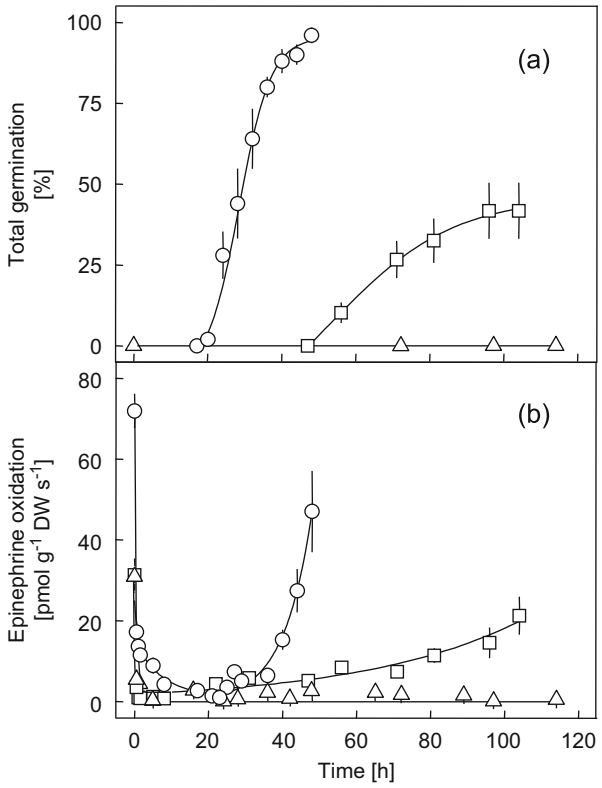

Fig. 3. Effect of artificial aging on (a) TG and (b) rates of extracellular $O_2^{\bullet -}$ production by non-aged seeds (circles), seeds that had been aged for 29 d (squares) and 102 d (triangles). No further germination occurred after the last data point in each curve. Values are given as ± SE, $n = 5$.

Table 2
Effect of the POX inhibitors KCN and NaN_3, and the NADPH oxidase inhibitor DPI on apoplastic $O_2^{\bullet -}$ production by *Pisum sativum* seedlings 48 h after the onset of imbibition. Inhibitors were added to the assay mixture together with the epinephrine and $O_2^{\bullet -}$ production was detected after 30 min. Data labelled with the same letters do not differ significantly ($P < 0.001$). Values are given ± standard error, $n = 5$.

Inhibitor	$O_2^{\bullet -}$ production (pmol g^{-1} DW s^{-1})
Control	5.0 ± 0.6 (100%)[a]
KCN (1 mM)	0.3 ± 0.1 (6%)[b]
NaN_3 (1 mM)	3.9 ± 0.8 (78%)[a]
DPI (10 μM)	3.8 ± 0.3 (76%)[a]

Table 3
Effect of temperature on extracellular $O_2^{\bullet -}$ production during the initial imbibition of dry peas. Superoxide production was detected as epinephrine oxidation after 30 min of incubation. Data labelled with the same letters do not differ significantly ($P < 0.001$). Values are given ± standard error, $n = 5$.

Temperature (°C)	$O_2^{\bullet -}$ production (pmol g^{-1} DW s^{-1})
10	24.2 ± 2.2[a]
20	43.9 ± 4.7[b]
25	69.4 ± 7.5[c]
30	91.7 ± 13.9[d]

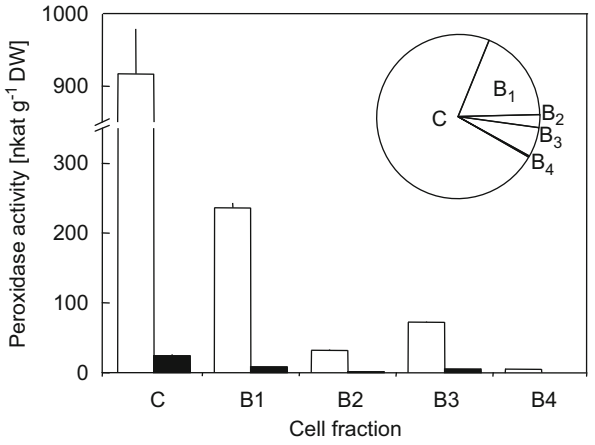

Fig. 4. ECPOX activity in cell wall fractions of radicles (white bars) and cotyledons (black bars) of *Pisum sativum* seedlings 48 h after the onset of imbibition. Fractions derived from seed coats had very low activities, between 0.03 ± 0.01 nkat g^{-1} DW (minimum) and 0.3 ± 0.01 nkat g^{-1} DW (maximum), and are not shown. C, crude cell fraction; B1, enzymes loosely bound to the cell wall, e.g. by hydrogen bonds; B2, enzymes bound by hydrophobic interactions; B3, enzymes bound by ionic bonds; B4, enzymes bound by covalent linkages. Inset, percentage of ECPOX activity in radicle cell fractions. Values are given as ± SE, $n = 5$.

Temperature significantly increased extracellular epinephrine oxidation during early imbibition (Table 3).

Radicle emergence and elongation were strongly correlated with increasing ECPOX activity (Fig. 1b). Peroxidase activity in cell fractions occurred mostly in the radicles (Fig. 1), and here was mostly in the crude cell fraction C (Fig. 4). However, ECPOX were also located in cell walls, and were associated mostly with the loosely and ionically bound fractions (fractions B1 and B3).

In-gel staining with NBT showed that $O_2^{\bullet -}$-producing enzymes with apparent molecular masses of approximately 210, 93, 85 and 50 kDa were mainly located in fraction B1 (Fig. 5a), with only trace activities in B2 (not shown) and B3 (both c. 93 kDa). The $O_2^{\bullet -}$-producing enzymes could readily use NADH as a reductant, but incubating gels for 15 h with NADPH revealed small amounts of $O_2^{\bullet -}$ production, corresponding to the same molecular masses as those with NADH (data not shown). Peroxidase visualization with guaiacol (Fig. 5b) produced a single band with molecular masses between 85 and 75 kDa in the B1 and B3 fractions. The position of these bands was confirmed using alternative POX substrates, including ABTS (not shown). TMB, a stain for haem groups, visualized proteins with similar molecular masses to those visualized by guaiacol in B1 and B3 (Fig. 5c). Lipoxygenase visualization using linolenic (not shown) or linoleic acid as a substrate revealed a single band in the B1 fraction with a molecular mass of *c.* 110 kDa (Fig. 5d).

Discussion

This study provides insights into the relationships among germination, viability loss during seed aging and the occurrence and roles of extracellular $O_2^{\bullet -}$ production in seeds. In *P. sativum* seeds and seedlings, specific stages of development or loss of viability correlate with changes in ROS production. Within the first 30 min of imbibitions, a burst of H_2O_2 production and epinephrine oxidation occurred (Fig. 1a, b). This initial oxidative burst was short-lived and did not reoccur after repeated wetting and drying cycles, suggesting that the enzyme(s) responsible for it and/or enzyme reductants were removed or deactivated during the first imbibition. While we could detect some $O^{\bullet -}$ by EPR (Fig. 2) and the epinephrine assay, experiments with SOD, inhibitors and XTT (Table 1) suggested that little $O_2^{\bullet -}$ was actually present. The initial oxidative burst decreased significantly with temperature, so could have been enzymatic or due to leakage of a substrate that oxidized epinephrine more slowly at lower temperatures (Table 3). Therefore, the initial oxidative burst probably comprised a mixture of H_2O_2, small amounts of $O_2^{\bullet -}$ and other unidentified molecules, which can leach from cells during imbibition and oxidize epinephrine. By contrast, the second increase in extracellular redox activity clearly involved $O_2^{\bullet -}$ and was strongly correlated with radicle elongation (Fig. 1a). The epinephrine, XTT, SOD and EPR assays all confirmed the identity of $O_2^{\bullet -}$. De-coated and whole seeds both acquired the ability to produce $O_2^{\bullet -}$ only after radicle protrusion had started (Fig. 1a and c), suggesting that the cotyledons were not involved in $O_2^{\bullet -}$ production. Unlike the first oxidative burst, the second ROS increase did not include H_2O_2. Although its presence was expected due to $O_2^{\bullet -}$ dismutation, H_2O_2 may have been broken down to H_2O by the abundant ECPOX. Furthermore, aging treatments reduced TG and extracellular $O_2^{\bullet -}$ production (Fig. 3) in a similar way as desiccation did in *Castanea sativa* embryonic axes (Roach et al., 2008, 2010). All the evidence presented (Figs. 1–3) suggests that the gradual increase in the ability of germinating seeds to produce $O_2^{\bullet -}$ correlated with radicle growth, consistent with the roles of $O_2^{\bullet -}$ in cell division, growth and differentiation. In addition, imbibed seeds and young seedlings will be attractive to seed predators due to the seed storage compounds, and thus highly vulnerable to pathogen attack. ROS can be directly toxic to pathogenic microorganisms and may also activate defense-related genes (Desikan et al., 2001). Hence, both the initial oxidative burst and the sustained $O_2^{\bullet -}$ production by seedling radicles could play a role in pathogen defense, and together with the developmental roles of ROS, may contribute to successful seedling establishment (Fig. 6).

Results obtained from experiments with inhibitors initially suggested that ECPOX were responsible for the second, more gradual increase in $O_2^{\bullet -}$ production during radicle elongation in pea seedlings (Table 2). The increase in extracellular $O_2^{\bullet -}$ production during early seedling growth was correlated with an increase in ECPOX activity (Fig. 1b) and with the emergence and growth of the radicle (Fig. 1b, c), where most POX activity was

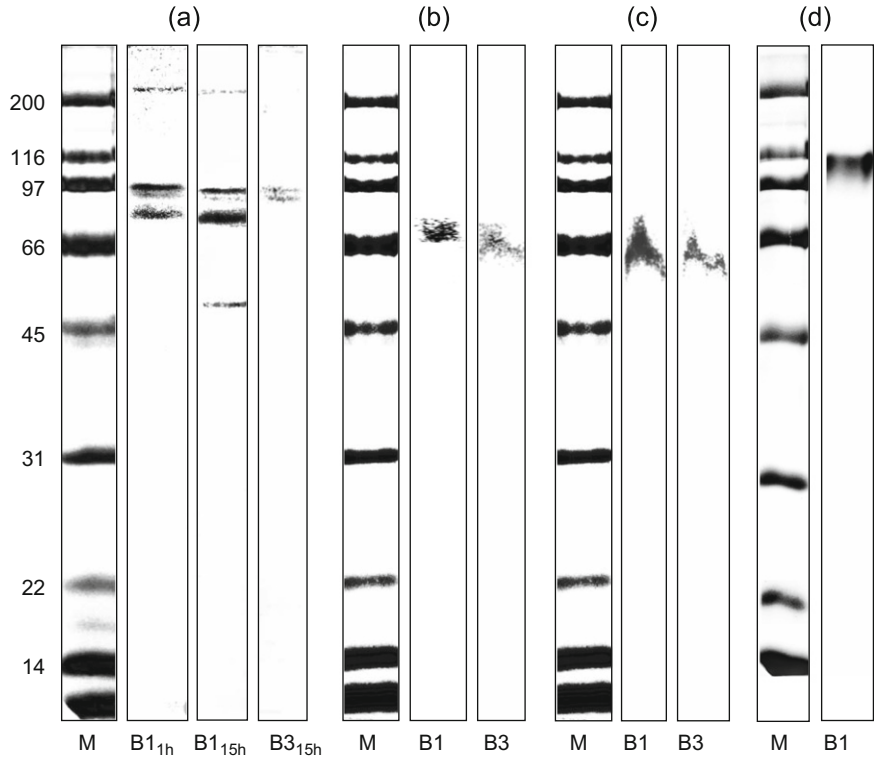

Fig. 5. Electrophoretic characterization of $O_2^{\bullet-}$ production, ECPOX and LOX activity in radicle cell wall fractions B1 and B3 of *Pisum sativum* seedlings 48 h after the onset of imbibition. (a) Superoxide production visualized by NBT with NADH for 1 h and 15 h for cell wall fraction B1 (proteins loosely bound to the cell wall) and 15 h for B3 (proteins bound by ionic bonds), (b) ECPOX staining with guaiacol, (c) haem-containing proteins stained with TMB; ECPOX and haem-containing proteins were absent from B2 and (d) LOX staining with linoleic acid in fraction B1. Other fractions did not stain (not shown).

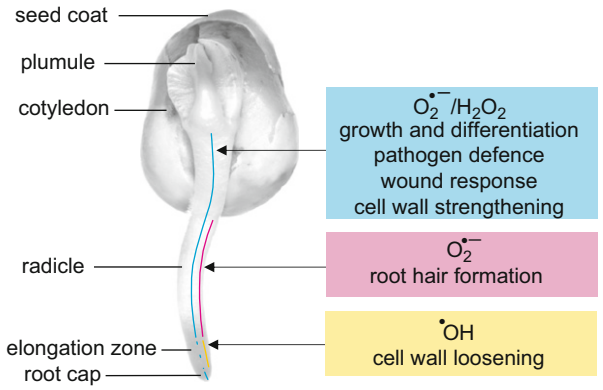

Fig. 6. Putative roles of extracellular ROS in *Pisum sativum* seedlings. Data presented here suggest that in *P. sativum* extracellular ROS play important roles at the time of early imbibition (Fig. 1) and further germination. In other seeds such as cress seeds, $^{\bullet}OH$ is important for endosperm weakening prior to germination (Müller et al., 2009) while its role in cell wall-loosening in pea seeds could be confined to the zone of cell elongation in the growing root. Both H_2O_2 and $O_2^{\bullet-}$ are required for growth, development and pathogen defense, and $O_2^{\bullet-}$ has also been implicated in root hair formation. The dotted line indicates low rates of $O_2^{\bullet-}$ production in the elongation zone (see Fig. 2). ROS-producing enzymes may include ECPOX, NADPH oxidases and lipoxygenases. See text for further details.

located (Figs. 2b, 4). Electrophoretic analyses of fractionated embryonic axes suggested the presence of a 75–85 kDa ECPOX in the "B1" (loosely bound) and "B3" (ionically bound) fractions. Unexpectedly, NBT staining of the gels showed that most $O_2^{\bullet-}$ production came from enzymes with molecular masses of 85 and 97 kDa (Fig. 5a), and neither was haem-containing (Fig. 5c). However, the existence of both POX activity and $O_2^{\bullet-}$-production in the B3 fraction suggests that POX could have contributed, as was clearly demonstrated for the recalcitrant seeds of *Castanea sativa* (Roach et al., 2010). Further electrophoretic investigations demonstrated the presence of apoplastic LOX (Fig. 5d), which can also produce $O_2^{\bullet-}$ (Roy et al., 1994). While the molecular masses of the $O_2^{\bullet-}$-producing enzymes did not precisely correspond to that of LOX (Fig. 5d), other LOX isoforms may have been present that used fatty acids other than linoleic or linolenic acid as substrates. In pea roots, LOX have been shown to contribute to the defense mechanisms against infection by pathogens (Leone et al., 2001). NAD(P)H oxidases have also been suggested as a source of $O_2^{\bullet-}$. Recently, Schopfer et al. (2008) described a NAD(P)H oxidase isolated from the plasma membranes of soybean with an identical molecular mass (85 kDa) to one of the $O_2^{\bullet-}$ producing bands found here (Fig. 5). Although plant NAD(P)H oxidases are normally considered to be membrane-bound rather than apoplastic enzymes (Sagi and Fluhr, 2006), moderate inhibition of the second increase by DPI (Table 2) suggests that they may have contributed to overall $O_2^{\bullet-}$-production. Results present here do not provide unequivocal proof for the identity of the $O_2^{\bullet-}$-producing enzymes, but roles for ECPOX, LOX or NAD(P)H oxidases cannot be excluded. More detailed analyses will be needed to determine the precise mechanisms of $O_2^{\bullet-}$ production in pea seeds.

In summary, the work presented here shows that, in germinating pea seeds, imbibition is accompanied by an immediate burst of redox activity, and later on, radicle emergence corresponds to a more gradual increase in extracellular $O_2^{\bullet-}$ production, both of which could be related to pathogen defense. Controlled extracellular ROS production plays important roles in many developmental and physiological processes in plants in general (Mika et al., 2004), and in particular in seeds (El-Maarouf-Bouteau and Bailly, 2008) (Fig. 6). The simultaneous increase in $O_2^{\bullet-}$ and ECPOX activity in the radicles of *P. sativum* seedlings

could result in the production of $^{\bullet}OH$ radicals required for cell wall loosening and subsequent cell elongation (Dunand et al., 2007; Kukavica et al., 2009). Importantly, $O_2^{\bullet -}$ also plays a role in developmental processes and is involved in the signaling implicated in cell elongation, cell division and differentiation (Gapper and Dolan, 2006). This paper provides clear evidence that $O_2^{\bullet -}$ production is an important component of seed germination, seedling growth and development, but further studies are needed to unravel the significance of the entire ROS signaling network and the precise contribution of the various ROS implicated.

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