Journal of Plant Physiology 167 (2010) 805–811

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

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

Ilse Kranner [a], Thomas Roach [a,b], Richard P. Beckett [c,*], Claire Whitaker [c], Farida V. Minibayeva [d]

[a] Seed Conservation Department, Royal Botanic Gardens, Kew, Wakehurst Place, Ardingly, West Sussex RH17 6TN, UK
[b] Research Department of Genetics, Evolution and Environment, University College London, Gower Street, London WC1E 6BT, UK
[c] School of Biological and Conservation Sciences, University of KwaZulu-Natal, Private Bag X01, Pietermaritzburg, Scottsville 3209, Republic of South Africa
[d] Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, P.O. Box 30, Kazan 420111, Russian Federation

ARTICLE INFO

Article history:
Received 10 November 2009
Received in revised form
9 January 2010
Accepted 11 January 2010

Keywords:
Apoplast
Hydrogen peroxide
Peroxidase
Superoxide

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

* Corresponding author. Tel.: +27 33 2605141; fax: +27 33 2605105.
E-mail address: rpbeckett@gmail.com (R.P. Beckett).

0176-1617/$ - see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2010.01.019

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

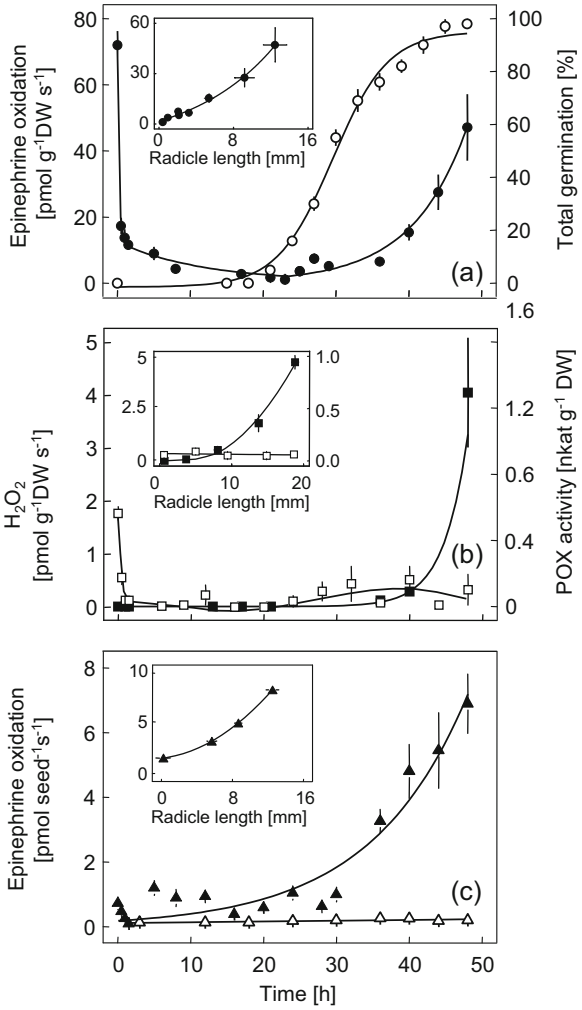

Fig. 1. Rates of extracellular $O_2^{\bullet -}$, assessed as epinephrine oxidation, and H_2O_2 production, and activity of released ECPOX during seed germination and early seedling development in *Pisum sativum*. Epinephrine oxidation during imbibition could not be exclusively attributed to $O_2^{\bullet -}$ (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, $n=5$.