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Mathematically combined half-cell reduction potentials of low-molecular-weight thiols as markers of seed ageing

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Abstract

The half-cell reduction potential of the glutathione disulphide (GSSG)/glutathione (GSH) redox couple appears to correlate with cell viability and has been proposed to be a marker of seed viability and ageing. This study investigated the relationship between seed viability and the individual half-cell reduction potentials (E_is) of four low-molecular-weight (LMW) thiols in *Lathyrus pratensis* seeds subjected to artificial ageing: GSH, cysteine (Cys), cysteinyl-glycine (Cys-Gly) and γ-glutamyl-cysteine (γ-Glu-Cys). The standard redox potential of γ-Glu-Cys was previously unknown and was experimentally determined. The E_is were mathematically combined to define a LMW thiol-disulphide based redox environment ($E_{\text{thiol-disulphide}}$). Loss of seed viability correlated with a shift in $E_{\text{thiol-disulphide}}$ towards more positive values, with a LD_{50} value of -0.90 ± 0.093 mV M (mean ± SD). The mathematical definition of $E_{\text{thiol-disulphide}}$ is envisaged as a step towards the definition of the overall cellular redox environment, which will need to include all known redox-couples.

Keywords: *Cysteine, cysteinyl-glycine, γ-glutamyl cysteine, glutathione, thiol–disulphide redox environment*

Abbreviations: *AsA, ascorbic acid; bis-γ-Glu-Cys, bis-γ-glutamyl-cystine; Cys-bis-Gly, cystinyl-bis-glycine; Cys-Gly, cysteinyl-glycine; DHA, dehydroascorbic acid; DHDT, 4,5-dihydroxy-1,2-dithiane; DTT, dithiothreitol; DW, dry weight;* $E_{cystine/2\ Cys}$*, half-cell reduction potential of the cysteine/cystine redox couple;* $E_{Cys\text{-}bis\text{-}Gly/2\ Cys\text{-}Gly}$*, half-cell reduction potential of the cysteinyl-glycine/cystinyl-bis-glycine redox couple;* $E_{bis\text{-}\gamma\text{-}Glu\text{-}Cys/2\ \gamma\text{-}Glu\text{-}Cys}$*, half-cell reduction potential of the γ-glutamyl-cysteine/bis-γ-glutamyl-cystine redox couple;* $E_{GSSG/2\ GSH}$*, half-cell reduction potential of the glutathione/glutathione disulphide redox couple;* E_i*, half-cell reduction potential of an individual low-molecular-weight thiol/disulphide redox couple;* $E_{thiol\text{-}disulphide}$*, the LMW thiol-disulphide redox environment, calculated by combining the* E_i*s of the investigated four individual thiol-disulphide redox couples; γ-Glu-Cys, γ-glutamyl-cysteine; GSH, glutathione (γ-glutamyl-cysteinyl-glycine); GSSG, glutathione disulphide;* LD_{50}*, value for an* E_i *that was lethal for half of the seed population; LMW, low-molecular-weight; NEM, N-ethylmaleimide; PCA, principal component analysis; PCD, programmed cell death; ROS, reactive oxygen species; TG, total germination; WC, water content*

Introduction

The tripeptide γ-L-glutamyl-L-cysteinyl-glycine (glutathione; GSH) is the most abundant low-molecular-weight (LMW) thiol in cells and is considered to be a major cellular redox buffer [1]. Glutathione homologues are found in certain plant families, such as homoglutathione (γ-L-glutamyl-L-cysteinyl-β-alanine) in the Leguminosae family [2], all of which share the common feature of a γ-glutamyl-cysteinyl moiety. Glutathione is an antioxidant that can directly scavenge reactive oxygen species (ROS), is involved in the reduction of lipid peroxides as a co-substrate

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for glutathione peroxidase (EC 1.11.1.9; glutathione: hydrogen peroxide oxidoreductase) [3] and participates in redox signalling [4–7]. Glutathione can reversibly form mixed protein disulphides with cysteine (Cys) residues of proteins, in a process known as glutathionylation [8]. Under conditions of oxidative stress, glutathionylation prevents the irreversible formation of inter- and intramolecular disulphide bridges which can disrupt protein structure and function [4]. Glutathionylation also occurs under non-stressful conditions and is likely to regulate protein function in cellular response pathways [9]. Other roles of GSH include the sequestration of heavy metals and xenobiotics. Many of its functions involve the oxidation of GSH to GSSG, which is reduced back to GSH by glutathione reductase (EC 1.8.1.7; glutathione: $NADP^+$ oxidoreductase) using NADPH as a reductant [10].

The GSH biosynthesis pathway appears to be the same in all organisms and occurs via two ATP-dependent steps. The first, catalysed by γ-glutamate-cysteine (γ-Glu-Cys) ligase [formerly, γ-glutamyl-cysteine synthetase; EC 6.3.2.2; L-glutamate: L-cysteine γ-ligase (ADP-forming)], forms γ-Glu-Cys and the second, catalysed by GSH synthase [EC 6.3.2.3; γ-L-glutamyl-L-cysteine:glycine ligase (ADP-forming)], forms GSH [3,11]. The degradation pathway is less well-characterized; γ-glutamyl transferases [formerly, γ-glutamyl transpeptidases; EC 2.3.2.2; (5-L-glutamyl)-peptide: amino-acid 5-glutamyltransferases] are the only enzymes capable of cleaving the unique γ-Glu-Cys peptide bond. In animal cells these enzymes are bound to the external cell membrane and GSH is exported from the cell and hydrolysed extracellularly. The resulting cysteinyl-glycine (Cys-Gly) dipeptide is hydrolysed by extracellular Cys-Gly dipeptidases (EC 3.4.11.2) and the free amino acids are transported into the cell where they can be used in GSH synthesis. This set of reactions is known as the γ-glutamyl cycle [11–13]. Evidence for this pathway of GSH degradation exists in plants [11], but may be restricted to the apoplast or vacuole [14]. In the cytosol GSH catabolism occurs via a γ-glutamyl transferase-independent pathway, initiated by γ-glutamyl cyclotransferase [EC 2.3.2.4; (γ-L-glutamyl)-L-amino-acid γ-glutamyltransferase (cyclizing)] [14].

The sensitivity of the thiol group to oxidation has led to the use of the GSH redox state as an indicator of oxidative stress within cells [1]. Oxidative stress arises as a result of ROS accumulation due to increased ROS formation or decreased ROS scavenging capacity. The 'Free Radical Theory of Ageing' states that the decline in function of an organism during ageing and the onset of age-related disorders is due to the accumulation of oxidative damage [15]. Ageing is often studied in humans and animals. However, plant seeds are equally good models in which to investigate the process of ageing. Seed can be artificially aged through exposure to elevated temperature and humidity [16] and the mechanisms of cell ageing, some of which may be similar in all aerobic organisms, can be studied without the need for animal experiments.

The majority of plant seeds, including those of the legume *Lathyrus pratensis*, are desiccation tolerant (termed 'orthodox') [17] and equilibrate with ambient humidity during 'maturation drying'. Consequently, the seed water content (WC) falls dramatically, which slows or stops metabolic activity within the seed and decreases the probability of metabolic ROS production. With decreasing water availability, the seed enters a state of suspended animation in which it can survive for long periods of time in the range from tens of years to decades. Nonetheless, seeds deteriorate over time and the products of ROS-induced damage such as oxidized proteins [18], inter-nucleosomal DNA fragmentation [19–22] and 4-hydroxynonenal formed through lipid peroxidation [23] are considered to be hallmarks of seed ageing and viability loss. Antioxidants such as GSH, ascorbate (AsA) and tocopherols scavenge ROS and protect against oxidative stress [3,6,7]. In desiccated orthodox seeds GSSG/2 GSH is the predominant antioxidant redox couple. In hydrated seeds and other hydrated plant tissues (such as leaves) AsA is present at high concentrations [3], whereas the dehydroascorbate (DHA)/AsA couple is apparently degraded during seed maturation drying [24–27]. In dry biological systems such as orthodox seeds, the concentrations of GSSG rise with storage time [8], indicative of ROS scavenging by GSH and its oxidation to GSSG in conjunction with the failure to re-reduce GSH. The resulting increase in the GSSG/GSH ratio leads to a shift in the half-cell reduction potential of the GSSG/2 GSH redox couple ($E_{GSSG/2\ GSH}$) to more positive values. This shift has been associated with loss of seed viability [21,28,29].

In addition to GSH, but usually at lower concentrations, most eukaryotic cells contain other LMW thiols such as Cys, Cys-Gly and γ-Glu-Cys [30]. Together with GSH these LMW thiols represent the major source of non-protein sulphur in cells and due to their nucleophilic thiol group all are highly reducing and readily participate in redox reactions. It is possible that the half-cell reduction potentials of these LMW thiol-disulphide redox couples also correlate with the physiological state of cells and tissues, but their contribution to the seed ageing process has not been investigated hitherto. Changes in $E_{GSSG/2\ GSH}$ have been proposed to act as a signal for the initiation of programmed cell death (PCD), in which nanoswitches [1] between LMW thiols and protein thiols could activate (meta)caspases [21]. Other LMW thiols could act in the same way

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and hence contribute to the induction of PCD responses.

In this paper, the concentrations of GSH, Cys, Cys-Gly and γ-Glu-Cys and their corresponding disulphides [i.e. GSSG, cystine, cystinyl-bis-glycine (Cys-bis-Gly) and bis-γ-glutamyl-cystine (bis-γ-Glu-Cys)] were determined during ageing of *Lathyrus pratensis* seeds and used along with standard reduction potentials to calculate the half-cell reduction potentials for each individual redox couple (E_i) according to the Nernst equation. The standard redox potentials of Cys, Cys-Gly and GSH have been previously reported, but that of γ-Glu-Cys was determined experimentally and reported for the first time in this study. We present evidence that distinct E_i zones specific to each redox couple can be defined at which half of the seed population was dead (LD_{50}). A zone of the mathematically determined $E_{thiol\text{-}disulphide}$ is also presented that correlates with LD_{50}.

Materials and methods

Seed material, ageing treatment and sample preparation

Seeds of *L. pratensis* were purchased from B&T World Seeds (France). Physical dormancy was removed by chipping the seed coat. Seeds were equilibrated to a WC of 12% over non-saturated lithium chloride in tightly sealed boxes (60% relative humidity) at 20 ± 1°C for ~5 weeks. Water contents were evaluated on a fresh weight (FW) basis. The dry weight (DW) was determined after heating at 103°C for 17 h and WC calculated using equation (1):

$$WC = \frac{(FW - DW)}{(FW)} \times 100 \quad (1)$$

Following equilibration, seeds were aged in sealed bottles at 50°C for 0, 5 and 10 weeks. Seed viability was assessed by germination tests on agar (1% (w/v)) at 25°C with an 8 h day (warm, white fluorescent light, 15 μmol/m^2/s)/16 h night cycle. Germination, defined as radical emergence of at least 2 mm, was scored until either all seeds had germinated or started to decompose. At intervals during ageing, seed samples were frozen in liquid nitrogen. The samples were then freeze-dried for at least 5 days in order to remove water through sublimation and thereby prevent unwanted redox reactions during storage (at −70°C prior to extraction) and during grinding. Throughout the study, analyses were conducted in parallel on (1) 30 'single seed' replicates and (2) four replicate 'populations', each consisting of pooled samples of 15 seeds. This approach was taken to assess the level of seed-to-seed variation within pooled samples and to determine whether there are significant differences between results obtained from analysis of single seeds compared to that of pooled samples [31,32]. Freeze-dried samples for population studies were ground to a fine powder in a liquid-nitrogen cooled Teflon container using a laboratory mill (Retsch MM200). The powder was stored at −70°C in humidity-proof vials until use. Dry seeds for single seed studies were ground in liquid nitrogen with mortar and pestle immediately prior to LMW thiol-disulphide extraction.

HPLC analysis of LMW thiols and disulphides

Cysteine, Cys-Gly, γ-Glu-Cys, GSH and their corresponding disulphides were extracted on ice in 0.1 M HCl from freeze-dried, finely-ground seed material and separated by reversed-phase HPLC (Jasco) [33]. Briefly, this assay uses fluorescence labelling of thiols by monobromobimane. 'Total' thiol and disulphide concentrations (e.g. GSH + GSSG) were determined after reduction of disulphides by dithiothreitol (DTT). For determination of disulphides, thiol groups were blocked with *N*-ethylmaleimide (NEM). Excess NEM was removed and the remaining disulphides were reduced with DTT and analysed as above. The GSH homologue, homoglutathione (γ-glutamyl-cysteine-β-alanine), found in some Leguminosae [2], was not detected.

Half-cell reduction potentials of LMW thiols and $E_{thiol\text{-}disulphide}$

Each redox couple forms an electrochemical half-cell, the reduction potential of which can be estimated. The half-cell reduction potential determines the voltage of the electrochemical cell using the concentrations of thiols and disulphides, taking into account deviations from standard conditions in terms of pH and temperature. The Nernst equation modified for

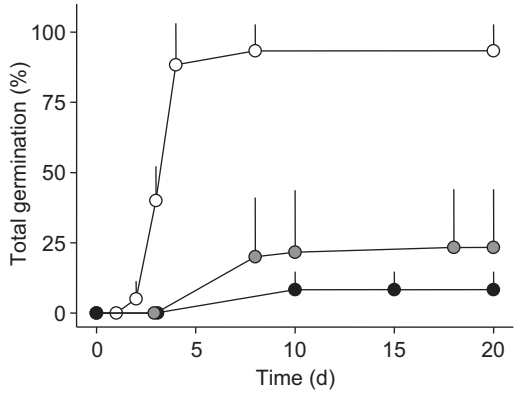

Figure 1. Accelerated ageing of *Lathyrus pratensis* seeds caused loss of viability, assessed by a drop in total germination (TG). Open symbols show TG of non-aged seeds (controls; '0 weeks') over a time course of germination testing on 1% agar. Grey and black symbols represent seeds that had been aged for 5 and 10 weeks, respectively. Data represent means ± SD ($n = 4$ replicates of 15 seeds per ageing treatment).

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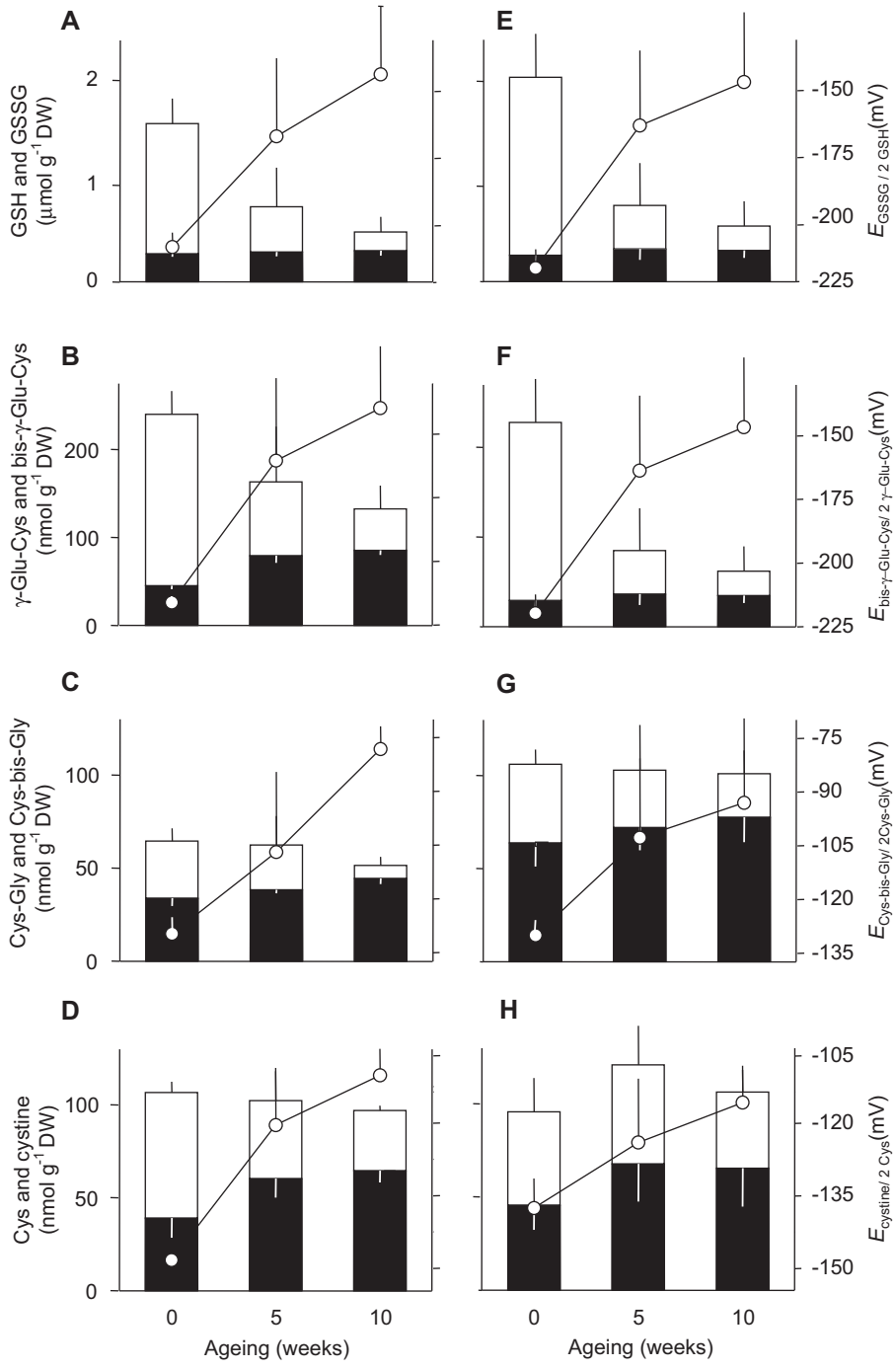

Figure 2. Ageing of *Lathyrus pratensis* seeds correlated with a decrease in the concentrations of LMW thiols (white bars) and an increase in their corresponding LMW disulphides (black bars). On the abscissas, '0', '5' and '10' weeks relate to 93%, 23% and 8% germination, respectively. (A–D) seed populations (means ± SD; $n = 4$ replicates of 15 seeds per ageing treatment); (E–H) single seeds (means ± SD; $n = 30$ individual seeds per ageing treatment). (A and E) GSSG/2 GSH; (B and F) γ-Glu-Cys/2 γ-Glu-Cys; (C and G) Cys-bis-Gly/2 Cys-Gly; (D and H) cystine/2 Cys (left ordinates). Lines and open circles represent half-cell reduction potentials (right ordinates).

determining the E_i of a LMW thiol redox couple is shown below (equation 2), along with the half-cell reaction (equation 3) [1].

$$E_i = E^{0'} - \frac{RT}{n\mathrm{F}} \ln \frac{[\text{LMW thiol}]^2}{[\text{LMW disulphide}]} \qquad (2)$$

$$2\text{LMW thiol} \rightarrow \text{LMW disulphide} + 2\mathrm{H}^+ + 2\mathrm{e}^- \qquad (3)$$

Where R is the gas constant (8.314 J/K/mol); T, temperature in K; n, number of transferred electrons; F, Faraday constant (9.6485×10^4 C/mol); $E^{0'}$, standard half-cell reduction potential of a thiol-disulphide redox couple at pH 7. Values used for $E^{0'}_{\text{GSSG/2 GSH}}$, $E^{0'}_{\text{Cys-bis-Gly/2 Cys-Gly}}$ and $E^{0'}_{\text{cystine/2 Cys}}$ were –240 mV [34], –226 and –226 mV [35], respectively. As the WC represents the mass of water as a

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percentage of the seed FW, the density of water (~1 g/mL) and the seed FW can be used to calculate the volume of water in the seed that is available for dissolution. Hence, the molar concentrations of LMW thiols and disulphides can be estimated. Whilst freeze-drying removes almost all water, some water will remain tightly bound to macromolecules (water sorption zone 1). However, this tightly bound water is not available for dissolution of solutes such as LMW thiols and disulphides and was therefore disregarded for the estimation of the volume of water used for calculating molar concentrations.

The biological standard redox potential (pH 7) of the bis-γ-Glu-Cys/2 γ-Glu-Cys redox couple ($E_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}}$) was determined in this study. Direct measurement of E_i by standard electrochemical methods is not possible due to formation of stable metal-thiolate complexes at electrode surfaces. Here, the $E_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}}$ was determined indirectly as previously described for other redox couples [36] by measuring the equilibrium constants for the reaction between the cystine/2Cys and 4,5-dihydroxy-1,2-dithiane (DHDT; 'oxidized DTT')/DTT redox couples, for which the E_is are known. Bis-γ-Glu-Cys (1 μM) was incubated for 3 h at 30°C in CHES buffer, with 10 μM cystine or DTT and different concentrations of Cys or DHDT (10 μM–500 mM). At the end of the reaction, the samples were treated with 0.1 M HCl to prevent any subsequent redox reactions. The analysis of thiols and their corresponding disulphides was performed as above. The equilibrium constant (K_{eq}) was calculated according to equation (4) [36]:

$$F_e = ([\text{Cys}]^2/[\text{cystine}])/(K_{eq} + ([\text{Cys}]^2/[\text{cystine}])) \quad (4)$$

where F_e is the ratio of bis-γ-Glu-Cys to γ-Glu-Cys at equilibrium. The standard redox potential was calculated using equation (5) (shown here for the cystine/2 Cys couple only) using biological standard redox potentials (pH 7) of –226 mV and –327 mV for the cystine/2 Cys and DHDT/DTT redox couples, respectively [35,37].

$$E^{0'}_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}} = E^{0'}_{\text{cystine/2 Cys}} - (RT/2\text{F}) \ln K_{eq} \quad (5)$$

The $E_{\text{thiol-disulphide}}$ was calculated using equation (6) [1] by summing the products of the E_is and reducing capacities of the four couples, as shown in equation (7).

$$E_{\text{thiol-disulphide}} = \sum_{i=1}^{n(\text{couple})} E_i \times [\text{reduced species}_i] \quad (6)$$

where E_i is the half-cell reduction potential for an individual redox couple and $[\text{reduced species}]_i$ is the concentration of the reduced species in that redox pair.

$$\begin{aligned} E_{\text{thiol-disulphide}} = {} & E_{\text{GSSG/2 GSH}} \times [\text{GSH}] \\ & + E_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}} \times [\text{γ-Glu-Cys}] \\ & + E_{\text{Cys-bis-Gly/2 Cys-Gly}} \times [\text{Cys-Gly}] \\ & + E_{\text{cystine/2 Cys}} \times [\text{Cysteine}] \end{aligned} \quad (7)$$

Statistical analysis

Data were analysed for significance by one or two-way ANOVA in combination with least significant difference (LSD) post-hoc comparisons of means. Correlations were statistically assessed using Principal Component Analysis (PCA) based on the Pearson's matrix of correlations. Probit analysis was used to determine LD_{50} values for the E_is.

Results

The effect of ageing on LMW thiols and disulphides

Artificial ageing of *L. pratensis* seeds at 50°C and 12% WC, hereafter termed 'ageing', resulted in almost complete loss of viability within 10 weeks (Figure 1). Non-aged seeds were highly viable, showing a total germination (TG) of 93%. Ageing for 5 weeks caused a decline in viability to 23% TG, which fell further to 8% after 10 weeks of ageing.

Non-aged seeds contained 1.5 μmol/g DW GSH + GSSG, 240 nmol/g DW γ-Glu-Cys + bis-γ-Glu-Cys, 64 nmol/g DW Cys-Gly + Cys-bis-Gly and 107 nmol/g DW Cys + cystine (Figures 2A–D). Between 0 and 5 weeks of ageing, the GSH + GSSG content fell by 50% ($p < 0.05$) and γ-Glu-Cys + bis-γ-Glu-Cys by 30% ($p = 0.001$), whereas Cys + cystine and Cys-Gly + Cys-bis-Gly contents were unaltered. The concentrations of all LMW disulphides increased, except for GSSG. The greatest change was observed for bis-γ-Glu-Cys ($p < 0.001$), which increased by 75%, followed by cystine ($p < 0.01$), which increased by 50% between 0 and 5 weeks of ageing. No significant changes in LMW thiol and disulphide concentrations were found between 5 and 10 weeks of ageing.

The concentrations of LMW thiols and disulphides were similar in both the bulk population study (Figures 2A–D) and the single seeds study (Figures 2E–H), with the exceptions of Cys-bis-Gly and bis-γ-Glu-Cys, which had higher ($p < 0.001$) and lower concentrations ($p < 0.05$), respectively, in the single seed analyses compared to those observed in the population analyses. However, the changes in LMW thiols and disulphides concentrations on ageing followed the same pattern in both the population and single seed samples. In terms of intra-sample variation, the LMW thiol-disulphide concentrations varied more between individual seeds; for example, the GSH concentration ranged between 0.81–2.54 μmol/g DW in non-aged single seeds, compared to a range of 1.02–1.53 μmol/g DW between the population samples.

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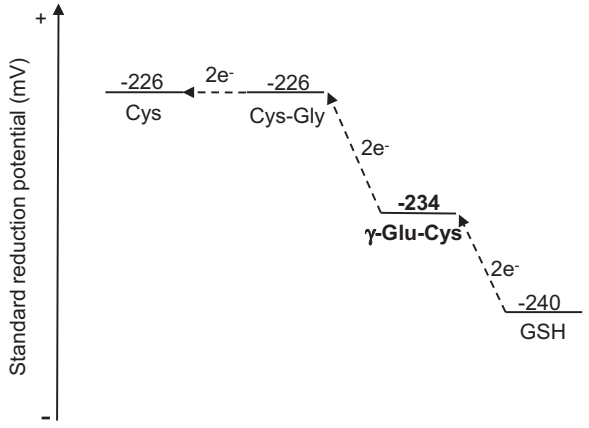

Figure 3. Standard reduction potentials of LMW thiols. The standard reduction potentials of GSSG/2 GSH, cystine/2 Cys and Cys-bis-Gly/2 Cys-Gly were known and that of bis-γ-Glu-Cys/2 γ-Glu-Cys ($E^{0'}_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}}$), indicated in bold font, was determined using different concentrations of the bis-γ-Glu-Cys/2 γ-Glu-Cys and cystine/2 Cys redox couples at equilibrium and by calculating their equilibrium constant (K_{eq}); see Methods for details.

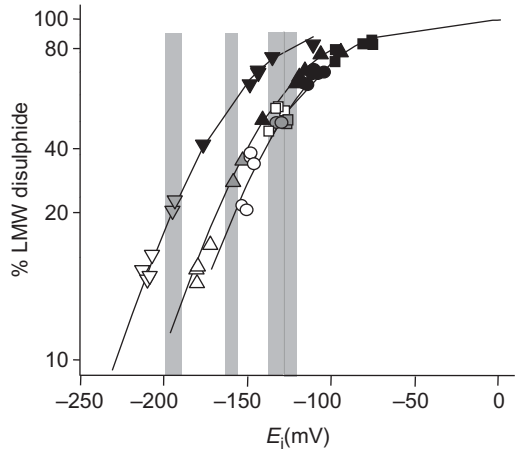

Figure 4. LMW disulphides (e.g. GSSG), expressed as a percentage of total corresponding LMW thiols + LMW disulphides (e.g. GSH + GSSG), plotted against the half-cell reduction potentials of (from the weakest to the strongest reductant) $E_{\text{GSSG/2 GSH}}$ (downwards triangles), $E_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}}$ (upwards triangles), $E_{\text{Cys-bis-Gly/2 Cys-Gly}}$ (squares) and $E_{\text{cystine/2 Cys}}$ (circles). Concentrations of LMW thiols found in *Lathyrus pratensis* seeds were [GSH] = 0.12–15.3 mM; [γ-Glu-Cys] = 0.12–3.7 mM; [Cys-Gly] = 0.02–0.18 mM; and [Cys] = 0.09–0.9 mM. Regression curves follow the equation:

$$\%\,\text{LMW disulphide} = \frac{100}{e^{\frac{(E^{0'}-E)\,nF}{[\text{LMW thiol}]RT}}+1}$$

with correlation coefficients of 0.99, 0.92, 0.91 and 0.80, respectively, for GSH/GSSG, γ-Glu-Cys/bis-γ-Glu-Cys, Cys-Gly/Cys-bis-Gly and Cys/cystine. Grey bars define the E_i values where half of the seeds had lost viability (LD_{50} values ± SD, assessed by probit analysis; $n = 4$ replicates of 15 seeds each per ageing treatment). These zones were –200 to –190, –164 to –154, –126 to –121 and –137 to –127 mV for $E_{\text{GSSG/2 GSH}}$, $E_{\text{γ-Glu-Cys/2 γ-Glu-Cys}}$, $E_{\text{Cys-bis-Gly/2 Cys-Gly}}$ and $E_{\text{cystine/2 Cys}}$, respectively. White, grey and black symbols show data from seeds with high viability (75–100% TG), intermediate viability (25–75% TG) and low viability (0–25% TG), respectively.

The greatest difference between minimum and maximum concentration values was observed for γ-Glu-Cys, which ranged from 0.04–343 nmol/g DW in single seeds aged for 5 weeks. Despite the observation of more extreme values in the single seed analyses, the standard deviations from the mean concentration values were only slightly greater than those obtained in the bulk population studies.

Effect of ageing on the E_i*s and* $E_{\text{thiol-disulphide}}$

The concentrations of LMW thiols and their corresponding disulphides were used to calculate the E_i values. The bis-γ-Glu-Cys/2 γ-Glu-Cys standard reduction potential was experimentally determined (see Methods) to be –234 mV (Figure 3). The E_is of all LMW thiol-disulphide redox couples increased (shifted towards more positive values) during ageing ($p < 0.001$). The increase was greatest for the redox couples with the highest reducing capacity (most negative standard redox potential), with $E_{\text{GSSG/2 GSH}}$ increasing by 68 mV; $E_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}}$ by 60 mV; $E_{\text{Cys-bis-Gly/2 Cys-Gly}}$ by 52 mV; and $E_{\text{cystine/2 Cys}}$ increased by 39 mV (Figure 2). Probit analyses produced LD_{50} values for $E_{\text{GSSG/2GSH}}$, $E_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}}$, $E_{\text{Cys-bis-Gly/2 Cys-Gly}}$ and $E_{\text{cystine/2 Cys}}$ of –190, –160, –121 and –133 mV, respectively (Figure 4) and a value of –0.90 ± 0.093 mV for the $E_{\text{thiol-disulphide}}$ (Figure 5). The percentage of outliers (e.g. values for seeds with a TG below 25% and $E_{\text{thiol-disulphide}}$ of -1.85 mV M) was 8.6% ($n = 102$). Again, redox potential and redox environment data did not differ significantly between single seed (Figures 2E–H and 5) and population studies (Figures 2A–D and 5). Glutathione concentrations significantly correlated ($R^2 \geq 0.82$, $p < 0.05$) with the concentrations of all other LMW thiols and showed an inverse correlation ($R^2 \geq -0.71$, $p < 0.05$) with the LMW disulphides studied. No correlation was found between GSH and GSSG, and GSSG was not correlated with any of the other LMW thiols or disulphides. The PCA plot (Figure 6) shows that the projection of the GSSG concentration corresponded to a vertical outlier as it had a small score distance (Factor 1) and a large orthogonal distance (Factor 2). On the other hand, the projections of the remaining concentrations of LMW thiols and their disulphides laid close to the PCA space where they were clearly separated into two groups. Disulphides clustered on the left and thiols on the right side of the PCA plot (Figure 6). The Pearson's correlation matrix showed that LMW thiols and disulphides lying close to the PCA space all significantly ($p < 0.05$) correlated with each other with R^2 values of at least 0.58. The E_i values all significantly ($p < 0.05$) correlated with each other with $R^2 \geq 0.912$.

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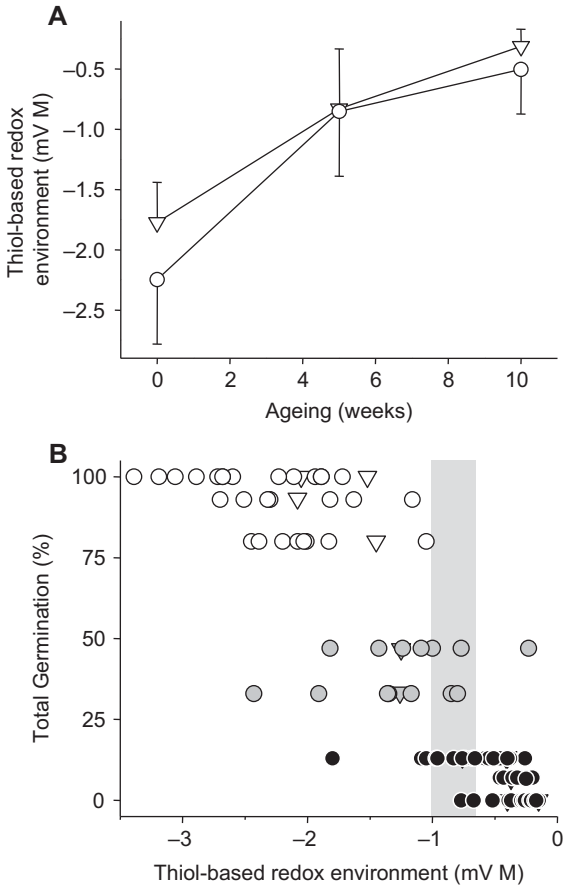

Figure 5. (A) The $E_{\text{thiol-disulphide}}$, defined by the mathematically combined values (see Methods) of $E_{\text{GSSG/2GSH}}$, $E_{\text{bis-γ-Glu-Cys/2 γ-Glu-Cys}}$, $E_{\text{Cys-bis-Gly/2 Cys-Gly}}$ and $E_{\text{cystine/2 Cys}}$ during ageing of *Lathyrus pratensis* seeds. Triangles represent seed populations (means ± SD; $n = 4$ replicates of 15 seeds each per ageing treatment) and circles represent individual seeds (means ± SD; $n = 30$ seeds per ageing treatment). (B) Data for the $E_{\text{thiol-disulphide}}$ plotted against TG. The grey bar between −1.025 and −0.688 mV represents the LD_{50} zone (means ± SD; $n = 30$ single seeds) in which 50% of all seeds had lost viability, assessed by probit analysis. Triangles show values for seed populations ($n = 4$ replicates of 15 seeds per ageing treatment) and circles represent individual seeds ($n = 30$ individual seeds per ageing treatment). White, grey and black symbols show data from seeds with high viability (75–100% TG), intermediate viability (25–75% TG) and low viability (0–25% TG), respectively.

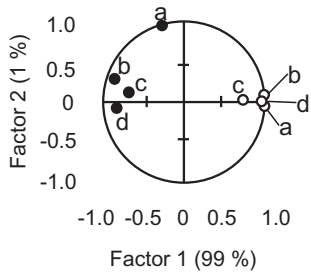

Figure 6. Principal component analysis (PCA) of data in Figure 1, showing that decreasing concentrations of LMW thiols and increasing concentrations of LMW disulphides are inter-correlated (significant at $p < 0.05$, using the Pearson's matrix of correlations, for all redox couples except for GSH/GSSG). Open circles represent LMW thiols and closed circles their corresponding disulphides: (a) GSH/GSSG; (b) γ-Glu-Cys/bis-γ-Glu-Cys; (c) Cys-Gly/Cys-bis-Gly; (d) Cys/cystine.

Discussion

Seed ageing resulted in a decline in GSH and γ-Glu-Cys concentrations and oxidation of Cys and Cys-Gly

As seeds lost viability, GSH concentrations decreased (Figure 2), indicative of degradation. In viable cells and whole plants GSH degradation is likely to play a role in the transport and delivery of Cys to specific sites. A decrease in the GSH pool is also indicative of cellular deterioration and death [21,38]. If GSH had been degraded, an increase in the concentrations of the products of degradation would be expected. This was not observed in this study. The concentrations of Cys-Gly + Cys-bis-Gly and Cys + cystine remained constant during ageing, whilst bis-γ-Glu-Cys + γ-Glu-Cys declined. It is possible that the products of degradation such as Cys are utilized in other pathways or participate in S-thiolation of proteins and, therefore, do not accumulate. Alternatively, the decline in GSH concentrations during ageing could be due to S-glutathionylation of Cys residues in proteins. S-glutathionylation involves the formation of mixed disulphides between protein thiol groups and GSH. It is a ubiquitous process in eukaryotes and also occurs in GSH-producing bacteria [8,39]. S-glutathionylation modulates the function and activity of regulatory, structural and metabolic proteins and the specificity of the process may be enhanced by enzymes such as glutaredoxins [8,40,41]. Under conditions of oxidative stress reversible protein S-glutathionylation occurs in order to protect protein thiols from irreversible oxidative damage [42,43]. Decline in GSH concentrations and formation of S-glutathionylated proteins have been reported to be early molecular events in the induction of cell death [44]. S-glutathionylation also protects GSH from irreversible oxidation (e.g. to sulphonic acid) and this may account for the observation that GSSG levels stayed constant during ageing. In contrast to GSSG, the concentrations of other LMW disulphides increased during ageing, indicative of oxidation of the corresponding LMW thiols. Whilst there is evidence of protein thiolation by Cys [45,46], there are no reports of γ-Glu-Cys participating in protein thiolation, so the decline in γ-Glu-Cys + bis-γ-Glu-Cys concentrations observed in this study are more likely due to degradation, which could proceed via γ-glutamylcyclotransferase to yield Cys and 5-oxo-proline, which can be subsequently used for protein thiolation and be converted to glutamate, respectively [14,47].

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Viability loss can be associated with E_i *zones that are distinct for each LMW thiol-disulphide redox couple*

It has previously been suggested that $E_{GSSG/2\ GSH}$ can be used to predict the point at which seed viability is lost in 50% of a seed population [21]. This suggestion was made in agreement with that of Schafer and Buettner [1] who proposed that in human cells, changes in $E_{GSSG/2\ GSH}$ are representative of the intracellular redox environment and that a change in $E_{GSSG/2\ GSH}$ to a zone of −180 to −160 mV corresponds to a physiological state at which PCD is initiated. In the absence of the DHA/AsA redox couple in dry seeds [25,27] and due to the stability of the disulphide bridge in GSSG, the GSSG/2 GSH couple forms the major cellular redox buffer. For human cells, $E_{GSSG/2\ GSH}$ was considered an intracellular redox buffer [1]. However, in plants GSH is the major transport form of reduced sulphur and is transported in the vascular bundles. Hence, in plants GSH occurs extracellularly in high concentrations. Therefore, we suggest that the terms 'redox buffer' and 'redox environment' are used for plants rather than 'intracellular redox buffer' and 'intracellular redox environment'. This study showed that not only $E_{GSSG/2\ GSH}$ but also $E_{bis\text{-}\gamma\text{-}Glu\text{-}Cys/2\ \gamma\text{-}Glu\text{-}Cys}$, $E_{Cys\text{-}bis\text{-}Gly/2\ Cys\text{-}Gly}$ and $E_{cystine/2\ Cys}$ shifted towards more positive values during seed ageing. The shift in E_i towards more positive values resulted from a decline in the GSH + GSSG and γ-Glu-Cys + bis-γ-Glu-Cys concentrations along with decreased thiol–disulphide ratios, whereas Cys-Gly + Cys-bis-Gly and Cys + cystine concentrations were maintained during ageing, but there was a shift from the thiol to the disulphide forms. The increase of $E_{GSSG/2\ GSH}$ with loss of seed viability supported the observations of previous studies [1,21,28,29] and validated the use of $E_{GSSG/2\ GSH}$ as a viability marker. The LD_{50} zone determined for $E_{GSSG/2\ GSH}$ was narrower and slightly more negative (by ~ 10 mV) than that previously determined [21] in which a range of species undergoing different stress treatments were used and a much larger sample size, which resulted in a broader LD_{50} zone that was applicable to a greater number of species and treatments. The evaluation of an LD_{50} zone for a single plant species and a particular stress treatment may result in a narrower zone that is more specific. In this study individual LD_{50} zones for $E_{bis\text{-}\gamma\text{-}Glu\text{-}Cys/2\ \gamma\text{-}Glu\text{-}Cys}$, $E_{Cys\text{-}bis\text{-}Gly/2\ Cys\text{-}Gly}$ and $E_{cystine/2\ Cys}$ were defined and this allowed these E_is to be used individually and together as indictors of seed viability.

Changes in $E_{thiol\text{-}disulphide}$ *during ageing of* Lathyrus pratensis *seeds*

The $E_{thiol\text{-}disulphide}$ was defined by summing the products of the E_is and the concentrations of the thiol species of each LMW thiol-disulphide redox couple. The $E_{thiol\text{-}disulphide}$ became more positive during ageing and this correlated with viability loss. A LD_{50} zone for the $E_{thiol\text{-}disulphide}$ could be defined as the point at which 50% of seeds had lost viability (0.90 ± 0.093 mV; Figure 5). Due to the highest molar concentration of GSH, $E_{GSSG/2\ GSH}$ had the greatest effect on $E_{thiol\text{-}disulphide}$. The contribution of the other E_is was calculated using equation (6) to estimate $E_{thiol\text{-}disulphide}$ with and without GSSG/2 GSH. Due to the lower concentrations of Cys and the dipeptide thiols, other E_is made a smaller contribution, which amounted to 15% in non-aged seeds and increased to 25% in seeds aged for 10 weeks. Therefore, although their effect on $E_{thiol\text{-}disulphide}$ is relatively small, they are worth taking into account, especially under conditions of oxidative stress, when other LMW thiols have a relatively greater impact as GSH declines. The $E_{thiol\text{-}disulphide}$ could be useful as an indicator of viability, especially for organisms with altered GSH metabolism, for example GSH knockdown mutants. Defining the redox environment in this way allows the combined effect of a number of redox couples to be quantified. This study concentrated on LMW thiols, because in dry orthodox seeds these are likely to have the greatest effect, yet other redox couples such as NADP/NADPH and DHA/AsA could also be included mathematically (by extending equation 7), to build a greater overall picture of the cellular redox environment. This could be useful for comparative studies between cell types, organs and species, because the contribution of individual redox couples to the cellular redox environment may vary on a cell-, organ- or species-specific basis.

Summary

Low-molecular-weight thiols play key roles in redox signalling and protect protein thiols from oxidative modification. Changes in the redox environment may promote thiolation processes, which could regulate protein activity and signalling pathways in response to increased ROS concentrations. In ageing seeds, LMW thiols appear to significantly contribute to the cellular redox environment and are key indicators of the redox changes taking place within cells during ageing. The E_is of each LMW thiol-dislphide redox couple define thresholds (LD_{50} value) at which half of the population has died, enabling the use of individual E_is as seed viability markers and they can also be combined mathematically to define $E_{thiol\text{-}disulphide}$.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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