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REGULAR ARTICLE

Wet-dry cycling extends seed persistence by re-instating antioxidant capacity

Rowena L. Long · Ilse Kranner · F. Dane Panetta · Simona Birtic · Steve W. Adkins · Kathryn J. Steadman

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Abstract Seeds in the field experience wet-dry cycling that is akin to the well-studied commercial process of seed priming in which seeds are hydrated and then re-dried to standardise their germination characteristics. To investigate whether the persistence (defined as *in situ* longevity) and antioxidant capacity of seeds are influenced by wet-dry cycling, seeds of the global agronomic weed *Avena sterilis* ssp. *ludoviciana* were subjected to (1) controlled ageing at 60% relative humidity and 53.5°C for 31 days, (2) controlled ageing then priming, or (3) ageing in the field in three soils for 21 months. Changes in seed viability (total germination), mean germination time, seedling vigour (mean seedling length), and the concentrations of the glutathione (GSH) / glutathione disulphide (GSSG) redox couple were recorded over time. As controlled-aged seeds lost viability, GSH levels declined and the relative proportion of GSSG contributing to total glutathione increased, indicative of a failing antioxidant capacity. Subjecting

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R. L. Long (✉)
School of Plant Biology,
University of Western Australia,
Crawley, WA 6009, Australia
e-mail: rowena.long@uwa.edu.au

R. L. Long · F. D. Panetta · S. W. Adkins
The Cooperative Research Centre for
Australian Weed Management,
Crawley, Australia

F. D. Panetta
e-mail: dane.panetta@deedi.qld.gov.au

S. W. Adkins
e-mail: s.adkins@uq.edu.au

I. Kranner · S. Birtic
Seed Conservation Department,
Royal Botanic Gardens, Kew,
Wakehurst Place,
Ardingly, West Sussex RH17 6TN, UK

I. Kranner
e-mail: i.kranner@kew.org

S. Birtic
e-mail: simona.birtic@cea.fr

F. D. Panetta
Queensland Department of Employment,
Economic Development and Innovation (DEEDI),
Alan Fletcher Research Station,
Sherwood, QLD 4075, Australia

S. W. Adkins
School of Land, Crop and Food Sciences,
The University of Queensland,
St Lucia, QLD 4072, Australia

K. J. Steadman
School of Pharmacy and School of Biological Sciences,
The University of Queensland,
St Lucia, QLD 4072, Australia
e-mail: k.steadman@uq.edu.au

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seeds that were aged under controlled conditions to a wet-dry cycle (to −1 MPa) prevented viability loss and increased GSH levels. Field-aged seeds that underwent numerous wet-dry cycles due to natural rainfall maintained high viability and high GSH levels. Thus wet-dry cycles in the field may enhance seed longevity and persistence coincident with re-synthesis of protective compounds such as GSH.

Keywords Ageing · *Avena sterilis* ssp. *ludoviciana* · Glutathione · Priming · Wet-dry cycling · Wild oat

Abbreviations

GSH	glutathione
GSSG	glutathione disulphide
MGT	mean germination time
RH	relative humidity
ROS	reactive oxygen species
SE	standard error
WC	water content

Introduction

Seeds in soils undergo wet-dry cycling in concert with fluctuations in the humidity of their surroundings (Wuest 2007). During rainfall and dew formation, soil surfaces can become saturated, and seeds with permeable seed coats imbibe water (Huang et al. 2008). If the duration of wetting is sufficient, seeds may then germinate, but if they are dormant, or if the temperature and light conditions are unsuitable for germination, the seeds will re-dry as moisture in their environment subsides (Batlla and Benech-Arnold 2006). The consequences of wet-dry cycling for the physiology of seeds and their subsequent persistence (defined as longevity *in situ*; Long et al. 2008) in the field are poorly understood, and are the focus of this study.

Wet-dry cycling experienced by seeds in the field is akin to the process of priming (Gonzalez-Zertuche et al. 2001), which is a commercial hydration-dehydration technique used to standardise the germination characteristics of a seed lot. The effects of priming on seed longevity are contentious, with some studies reporting protracted longevity following priming (Butler et al. 2009; Yeh and Sung 2008), and others reporting shortened longevity (Lin et al. 2005; Probert et al. 1991). One major factor influencing the consequences of priming for seed longevity is the physiological age of the seeds at the time of priming, such that longevity is promoted in seed lots that have lost vigour prior to wetting (Butler et al. 2009). It is possible that wet-dry cycling occurring during rain events in natural conditions may also extend the persistence of ageing seeds – a prospect that has implications for weed management and land restoration.

The mechanism by which priming enhances seed longevity is likely associated with antioxidants, which can protect and repair cellular components (Bailly et al. 1998; Yeh and Sung 2008). A major cause of seed deterioration is the accumulation of reactive oxygen species (ROS). ROS play significant roles in diverse processes such as stress signalling, gene expression, plant growth and development, but if not carefully controlled by antioxidants, they can accumulate and cause oxidative stress (Bailly 2004; Kranner and Birtic 2005; Foyer and Noctor 2009). Indicators of oxidative stress include changes in the activities of the antioxidants that scavenge ROS (Bailly et al. 2002; Goel et al. 2003; Kranner et al. 2006), and changes in membrane permeability (Mirdad et al. 2006; Spano et al. 2007) and lipid peroxidation (Goel and Sheoran 2003; Murthy et al. 2003). The occurrence and rate of these reactions are strongly influenced by the water content of seeds; whilst greater metabolic activity in wetter seeds is associated with more, potentially harmful, ROS being produced, water-soluble antioxidants are active and can scavenge ROS to prevent them accumulating to toxic levels. One water-soluble antioxidant system present in all cells, the glutathione system, shows particular promise as a correlate of seed viability loss (Kranner et al. 2006); as seeds age, glutathione (γ-glutamyl-cysteinyl-glycine; GSH) scavenges ROS that could otherwise accumulate and damage the cell, and GSH is oxidised to glutathione disulphide (GSSG) in the process (Colville and Kranner 2010). Whilst studies using seeds aged and primed in the laboratory have indicated a role for GSH in reinvigorating seeds (Bailly et al. 1998; Yeh and Sung 2008), whether or not the GSH/GSSG system is associated with extending the persistence of seeds undergoing wet-dry cycling in the field has yet to be reported.

Given that seeds in the soil seed bank undergo wet-dry cycling that is analogous to priming, we

reasoned that antioxidant capacity will decline in ageing seeds but will be reinvigorated by wet-dry events, and will be accompanied by an extension in seed longevity. To test this hypothesis, we aged and primed seeds of the global agronomic weed *Avena sterilis* subsp. *ludoviciana* in the laboratory and assessed their viability, seedling vigour, and contents of GSH and GSSG. Seeds recovered from a 21-month-long field-based burial trial were also tested to gauge whether patterns observed in the laboratory were consistent with field scenarios in a range of soil types.

Materials and methods

Seed materials

Florets of *Avena sterilis* ssp. *ludoviciana* were collected from a chickpea (*Cicer arietinum* L.) crop located between Millmerran and Goondiwindi, Queensland, Australia (S 28.19°, E 150.46°) on 20 October 2004. Florets were stored in the laboratory (approximately 50% relative humidity (RH) at 21±1°C) for 6 weeks prior to the start of experiments.

Seed ageing treatments

Three seed-ageing treatments were applied: 1) controlled ageing, 2) controlled ageing-plus-priming, and 3) field ageing.

Controlled ageing was conducted using a modified version of the test described in Long et al. (2008). Prior to controlled ageing, caryopses (hereafter referred to as seeds) were stored with florets intact at 9.2±0.1% water content (WC; dry weight basis) in vacuum-sealed foil bags in a −20±1°C freezer for 19 months. Vials containing 25 seeds were pre-equilibrated to 47.0±1.5% RH (370 gL^{-1} LiCl) at 20±1°C for 14 days in a sealed box to reach a final WC of 9.9±0.3%. The ageing test started when these vials were transferred to a second sealed box at 60.0±1.5% RH (290 gL^{-1} LiCl) and 53.5±0.5°C, which maintained WC at a similar level (9.7±0.3%). On days 0, 8, 16, 22, 27 and 31 of controlled ageing, vials were retrieved to assess WC gravimetrically (105°C for 24 h, two replicates), seed germination (four replicates) and glutathione redox status (four replicates).

For the controlled ageing-plus-priming treatment, seeds were first aged for 25 days (as described above) before being moved to a priming environment at 20±1°C for 6 days. Three treatments comprised: −1 MPa (99.6% RH at 20°C, 47% WC), −10 MPa (93.5% RH at 20°C, 27% WC) and unprimed (47% RH at 20°C, 11% WC) to simulate soil moisture conditions within and below the plant-available-water range of 0 to −1.5 MPa (Brady and Weil 1999). Seeds were primed using 5 mL polyethylene glycol solutions (PEG 8000, hereafter 'PEG'; −1 MPa=295 gL^{-1} deionised H_2O at 20°C; −10 MPa=974 gL^{-1}) applied to two discs of Whatman No. 1 filter paper in 90 mm Petri dishes. Seeds remained on the PEG solutions for 4 days, were rinsed briefly in a tea-strainer with deionised water, and then dried at 47% RH for 2 days to return them to their original WC. For the unprimed treatment, seeds were transferred to 47% RH and 20°C for 6 days. After 6 days (Day 31), half of the samples of the primed and unprimed treatments were retrieved for germination, glutathione redox state, and WC tests, whilst the remaining samples received a further 3 days of ageing at 60% RH and 53.5°C (to day 34). For comparison, a standard controlled ageing treatment was prepared, and samples were retrieved on Days 0, 25, 31 and 34 of ageing. For each treatment on each retrieval day, two replicates of 50 seeds were used for germination testing, two replicates of 25 seeds were used for moisture content testing, and four replicates of 25 seeds were used for analysis of glutathione redox status.

For field ageing, samples of approximately 750 seeds in their floret tissues were buried at 10 cm and exhumed according to Long et al. (2009). The soil pH was approximately 6, 7 and 8, respectively for the red light clay, sandy loam and black silty loam soils (Long et al. 2009). Soil moisture and temperature were monitored and ranged from 55% to 100% RH and 10°C to 40°C, depending on the soil, season, and rainfall events (Long et al. 2009). Additionally, the rainfall events for the site where all three soils were located were interpreted from data from the Australian Government Bureau of Meteorology (Fig. 1). For each soil and block, enough seed samples were buried to enable retrievals at 4.5, 9, 12, 17 and 21 months after burial. However, the number of filled seeds that were retrieved gradually declined during the burial trial. Visual inspection of soil samples indicated that some seeds had decomposed or germinated and died prior to

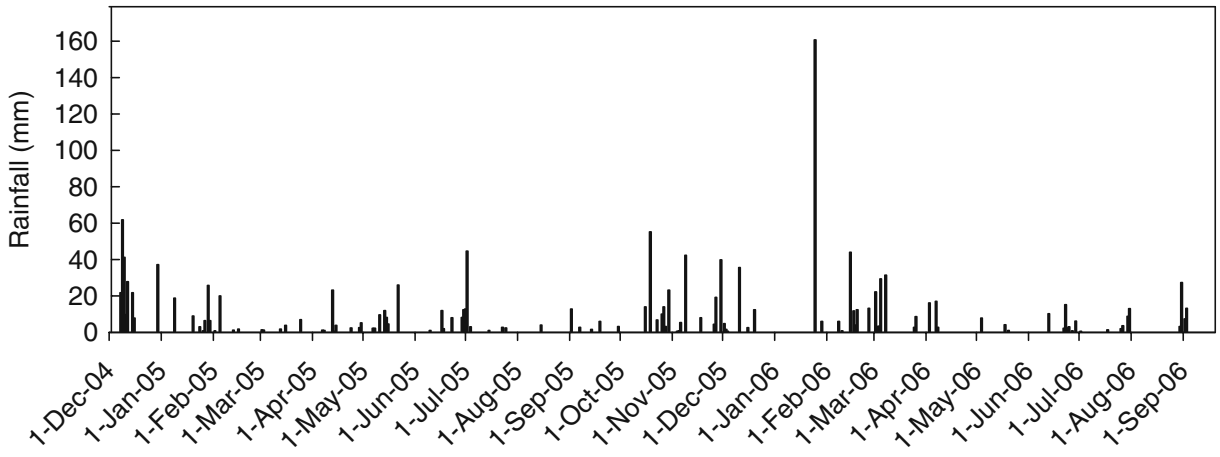

Fig. 1 Rainfall events experienced by seeds during the 21-month-long seed-burial experiment. Data were obtained from the Australian Government Bureau of Meteorology (2010)

emerging. For the black soil, no filled seeds were recovered at 12 months and subsequent retrieval times. Seeds were recovered throughout the 21 months for the red and sandy soils but at 21 months sufficient seeds for viability, vigour and glutathione testing were only recovered from one of four blocks. Thus, for field ageing, analyses reflect results for those seeds that had not fatally germinated or decomposed. Seeds were removed from their floret tissues prior to further testing.

Seed viability

Viability was assessed by a germination test and data are expressed as a germination percentage of filled seeds. Seeds were germinated at 20±1°C under constant white light (c. 50 $\mu mol\,m^{-2}s^{-1}$) on 1% water agar that contained 722 μM GA_3 to bypass dormancy so that lack of germination was related to lack of viability and not the presence of dormancy. Four replicates of 25 seeds were tested for controlled ageing samples, two replicates of 50 seeds for controlled ageing-plus-priming samples, and 25 field-aged seeds were tested if sufficient filled seeds were recovered from each block. Germination was defined by radicle protrusion of ≥0.5 mm and was scored daily for up to 30 days. Abnormal seedlings (those exhibiting premature shoot protrusion or stunted radicle growth) were excluded so that viability reflects the total number of normal germination events only. After 30 days, any seeds that had not germinated were cut-tested to assess final viability; seeds with intact, white embryos and endosperm were scored as viable, whilst those seeds that were bloated or discoloured were scored as non-viable.

Glutathione quantification

Extraction and quantification of GSH and GSSG were conducted following the protocols of Kranner and Grill (1996) and Kranner (1998). After retrieval at 4.5, 9, 12, 17 and 21 months, field-aged seeds were removed from floret tissues and dried in paper bags at 15% RH and 15°C for between 2 and 4 weeks. Each sample contained up to 25 seeds; due to seed decay in the field, samples were not available for some combinations of soil and retrieval time. Controlled-aged seeds were retrieved after 0, 8, 17, 22, 28 and 31 days of ageing. Controlled ageing-plus-priming samples were retrieved on Days 0, 25, 31 and 34. All samples were weighed and vacuum-sealed in a foil bag with 14 g dry silica gel and frozen at −80°C. Prior to extraction, samples were freeze-dried to remove free water, and ground to a fine powder with agate balls in a liquid nitrogen-cooled grinding capsule using a Retsch MM200 ball mill. Low-molecular-weight thiols were extracted from the resulting powder by the addition of 1 mL of 0.1 M HCl to 50±2 mg seed material (exact weight recorded). During the extraction process, samples were kept on ice to minimize unwanted reactions. Samples were vortexed for 20 s to combine, then centrifuged at 18,000*g* at 4°C for 40 min.

Thiols were analysed using reversed-phase HPLC (Jasco) in combination with fluorescence detection

(excitation wavelength=380 nm, emission wavelength=480 nm) according to the methods used in Kranner and Grill (1996). Glutathione was separated from other low-molecular-weight thiols: cysteine, cysteinyl-glycine and γ-glutamyl-cysteine. The sample concentrations of each component were calculated using calibration curves of standards. Standards of hydroxy-methyl-glutathione (γ-glutamyl-cysteinyl-serine; hmGSH) were also used to check for the presence of this glutathione homologue that is present in some Poaceae species, however it was not detected. Cyst(e)ine, cyst(e)inyl-glycine and γ-glutamyl-cyst(e)ine occurred in trace amounts in some samples only, and their accurate quantification was hindered due to their concentrations approaching the lower detection limits of this technique. Thus only results for GSH and GSSG are reported.

Statistical analysis

Generalized linear models (GLM) were used to test for the effects of ageing duration, hydration level, soil type, replicate or block, and their interactions, where relevant, on seed viability and quality, seedling vigour and GSH / GSSG content using controlled- and field-aged seeds. Germination data were arcsine-transformed. GLM analysis of viability data for field-aged seeds was weighted (1/N) to account for variation in numbers of seeds recovered. Standard errors (SE) were pooled in viability analyses, but SEs for individual time points are shown in figures. For significant effects only, subsequent comparisons of treatment levels within experiments were conducted using two-sample t-tests and adjusted for multiple comparisons (Bonferroni).

Results

Effects of controlled seed ageing on viability and vigour

Seeds subjected to controlled ageing in the laboratory declined from 100% viability at Day 0 to 16% at Day 31 (Fig. 2a, pooled SE=0.14). The main drop in germination occurred after Day 22, and this was coupled with seeds taking longer to germinate and seedlings growing more slowly. The 16% of seeds that were still able to germinate at Day 31 took an average time of 11 days to germinate, significantly slower than seeds aged for 0 to 22 days, which germinated in 4 to 6 days (Fig. 2b, $P<0.001$, pooled SE=0.4); seedlings germinating on Day 31 grew to <20 mm, instead of 50 to 70 mm (Fig. 2c).

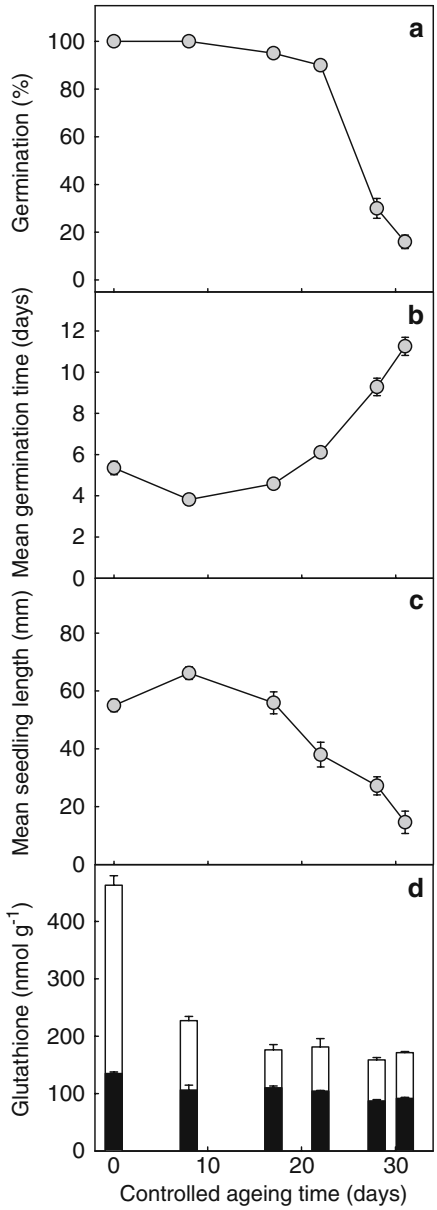

Fig. 2 The effects of controlled ageing (60% RH and 53.5°C for up to 31 days) of *A. sterilis* seeds on **a** seed viability (% germination), **b** mean germination time (MGT), **c** seedling vigour (mean seedling length 5 days after the first seed in each Petri dish had germinated) and **d** glutathione concentration (nmol g^{-1} dry weight; GSH, white bars; glutathione disulphide, GSSG, black bars). Data points show mean±SE (n=4 replicates of 25 seeds)

Effects of controlled seed ageing and wet-dry cycling on glutathione redox state

For seeds that were aged under controlled conditions, the concentrations of GSH, GSSG and total glutathione (GSH+GSSG) declined during the first 17 days of ageing, with the overall drop due primarily to a fall in GSH concentration that occurred between Day 0 and Day 8 ($P<0.001$; Fig. 2d).

To simulate a rainfall event, seeds that had been aged for 25 days in the controlled ageing environment were removed from it and subjected to a wet-dry cycle. When removed from the controlled ageing environment at Day 25 and primed at −1 MPa (a water potential within the normal plant-available-water range), the concentration of GSH increased significantly by Day 31 compared to seeds that were not primed ($P<0.001$; Fig. 3). Moreover, seed viability remained high at 98% even after returning to the ageing environment for a further 3 days. In contrast, seeds that were not primed continued to lose viability when removed from the ageing environment and viability loss was hastened when the ageing treatment was recommenced. Seeds primed at −10 MPa also exhibited enhanced GSH levels relative to unprimed seeds ($P=0.006$), and slightly higher germination (Day 31 $P=0.147$, Day 34 $P=0.056$). Thus priming to both −1 MPa and −10 MPa stimulated GSH production and recovered the germination capacity of the seeds.

Effects of field ageing on seed viability, vigour and glutathione redox state

For seeds buried in red and sandy soils, which retained high viability, the proportion of GSH relative to GSSG remained high (Fig. 4a, d and e) in all samples throughout the 21 months. The viability of seeds that could be retrieved from the black soil declined by about 35% after 9 months of ageing, but the loss of viability was not yet reflected in the concentrations of GSH and GSSG (Fig. 4a and f). The 60% of seeds that were able to germinate when retrieved from black soil at 9 months grew to only 30 mm in length in 5 days. In contrast, filled seeds

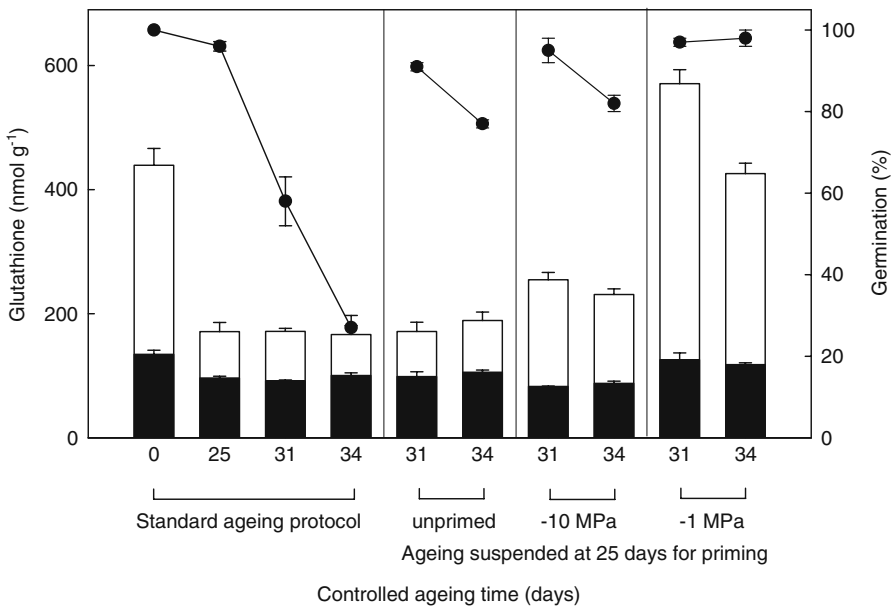

Fig. 3 The effect of priming on viability and glutathione redox state of *A. sterilis* seeds. Seeds were aged under controlled conditions (60% RH and 53.5°C for 34 days), as in Fig. 1, but ageing was suspended at Day 25 for 6 days for a subset of the seeds. Seeds were primed at −1 MPa or −10 MPa on polyethylene glycol for 4 days, to simulate a rain event, followed by drying (47% RH at 20°C for 2 days), then returned to the ageing environment on Day 31 for a further 3 days (concluding on Day 34). Unprimed samples were held at 47% RH and 20°C for 6 days then returned to the ageing environment. Glutathione concentration (GSH, nmol g^{-1} dry weight, white bars; GSSG, black bars), seed viability (% germination, closed circles). Data are mean±SE ($n=4$ replicates of 25 seeds for glutathione concentration; $n=2$ replicates of 50 seeds for viability)

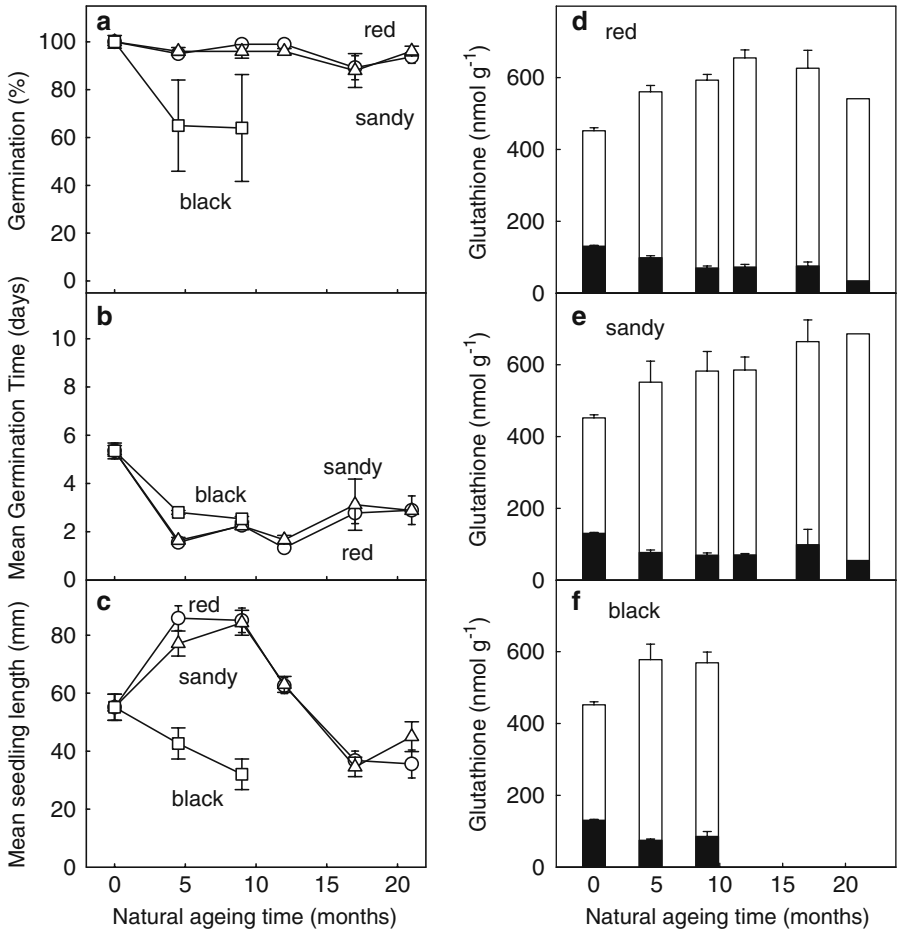

Fig. 4 The effect of field burial on **a** seed viability (% germination, from Long et al. 2009), **b** mean germination time, **c** seedling vigour (mean seedling length 5 days after the first seed in each Petri dish had germinated), and (**d**-**f**) glutathione concentration (nmol g^{-1} dry weight; GSH, white bars; GSSG, black bars) of *A. sterilis* seeds. Seeds were buried in (**d**, and circles in **a**-**c**) red soil, (**e**, and triangles in **a**-**c**) sandy soil and (**f**, and squares in **a**-**c**) black soil for 21 months. No seeds remained in the black soil **f** after 9 months. Data are mean±SE (n=4 replicates of 25 seeds each)

that were recovered from the red and sandy soils retained high viability (>80%) and seedlings grew to approximately 80 mm in 5 days (Fig. 4). Indeed, the effects of red and sandy soils on MGT and seedling vigour were indistinguishable (Fig. 4b and c; P=0.890 and 0.864). From 12 months onwards, no samples were recovered from the black soil. Also, fewer samples were available at 17 and 21 months for the red and sandy soils as no filled seeds were recovered from several blocks, and this hindered further assessment of the relationship between glutathione concentrations and seed viability in field environments.

Discussion

Ageing and priming seeds in the laboratory afforded insights into the effects of rainfall on the persistence of seeds in the field. When partially-aged seeds from the controlled ageing environment were subjected to a wet-dry cycle and then returned to the ageing environment, in simulation of a rainfall event, GSH levels mimicked those of field-aged seeds. GSH increased relative to GSSG in primed seeds, concomitant with increased seed viability and extended seed longevity. Together, these results suggest a role for wet-dry cycling in determining the persistence of seeds in the field through a mechanism that may involve recovery of the glutathione antioxidant system.

The degree of antioxidant repair and reinvigoration of seeds by wet-dry cycles may depend on the age and hydration state of the seeds, as determined by their environment. Classic studies by Osborne and coworkers demonstrated that seed ageing is associated with damage to nucleic acids and proteins, which can to some extent be repaired upon imbibition (Cheah and Osborne 1978; Sen and Osborne 1974). Consis-

tent with these early studies, our data suggest that the effectiveness or speed of such repair mechanisms depends on seed WC. The magnitude of the GSH increase in seeds primed at −1 MPa compared to the unprimed treatment suggests that GSH was synthesized rather than recovered from GSSG. Although less pronounced than at −1 MPa, GSH was also produced during priming at −10 MPa. These results extend the view of Vertucci and Farrant (1995) that repair processes occur when seeds are hydrated at −3 MPa and wetter, and are further supported by a recent study of *Digitalis purpurea* by Butler et al. (2009) in which the viability of aged seeds improved following priming to as low as −15 MPa. Moreover, our priming study afforded an insight into the effects of rainfall on seed persistence: the field-aged seeds received numerous rainfall events during 21 months of burial (Fig. 1), so we surmise that their sustained high GSH concentrations and high viability may have resulted from the priming effect of rainfall.

Whilst antioxidant capacity appears to contribute to seed persistence, some distinctions in the ageing conditions experienced by seeds in the burial trial and laboratory should be considered when interpreting these results. Firstly, the population subjected to field-burial decreased in size due to seeds germinating, deteriorating or being predated, so only the longer-lived sub-population was retrieved and therefore represented in field-aged samples (Ishikawa-Goto and Tsuyuzaki 2004). In contrast, no seed attrition occurred during the 30-day controlled ageing treatment, so a random sample of the entire population was taken each time. Secondly, seeds in controlled ageing conditions were maintained under stable conditions whereas seeds aged in the field were exposed to fluctuations in moisture and temperature. Sustained high temperature (53.5°C) during controlled ageing at a moderate WC (10 % WC at 60% RH) would favour cellular damage and limit its repair (Vertucci and Farrant 1995; Walters et al. 2005). In contrast, the field-aged seeds experienced moisture fluctuations between 55% and 100% RH, and a less extreme temperature range of 10°C to 40°C; differences in soil temperature and water content contributed to the differing effects of soil types on seed viability and vigour, with the black soil being the warmest and wettest, and thus displaying faster losses in seed viability and vigour than the red and sandy soils (Long et al. 2009). Nevertheless, the similarity of viability and glutathione profiles of the field-aged seeds with those of the priming treatments indicates that those seeds that do persist in the field may be recovering their antioxidant capacity during rainfall events.

The implications of this study are pertinent for weed management and land restoration, where the longevity and dynamic nature of seed banks are important considerations. Here we suggest that the longevity of seeds may recover and even be extended by wet-dry cycling resulting from rain events. Estimating the persistence of weed seeds in the soil is a major challenge for policy makers and practitioners involved in containing and eradicating weeds, and those using seeds stored in soil for rehabilitation of mined and degraded lands (Panetta 2007; Rosef 2008). The traditional method for assessing seed persistence is the seed burial trial, which has limitations because it is time-consuming and the results may not be relevant beyond the specific soil and climate conditions of the study site (Ishikawa-Goto and Tsuyuzaki 2004; Long et al. 2009). Recent attempts to accelerate predictions of seed persistence using laboratory-based controlled-ageing tests appear promising (Long et al. 2008; Long et al. 2009; Schoeman et al. 2010); however, they do not account for a potential extension in seed longevity that may occur with wet-dry cycling (as found in this study), so may underestimate persistence, such as in Panetta (2009). Further studies are needed to determine if there is an optimal number of wet-dry cycles to extend longevity, and if a decline in seed viability after the optimum number of cycles is associated with antioxidant breakdown.

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