RESEARCH ARTICLE

Aerobic environment ensures viability and antioxidant capacity when seeds are wet with negative effect when moist: implications for persistence in the soil

Running head: Aerobiosis versus anaerobiosis in wet or moist seed viability

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Abstract

Interchangeable effects of temperature, moisture content and oxygen on seed longevity have been mostly examined to estimate seed viability during long-term dry storage whereas few experiments studied seed viability under near-natural conditions to evaluate seed persistence in the soil. To this end, we artificially aged seeds of *Ranunculus baudotii*, a hydrophyte widely distributed in temporary ponds constituting abundant soil seed bank. *S*eeds were exposed to controlled ageing at three different relative humidities (RH) under both aerobic and anoxic conditions. Their viability, water content, membrane damage, oxidative stress and antioxidant enzymatic defence activity were evaluated. Seed survival was longer at higher relative humidity (97% RH), and lowest at a relative humidity (90% RH) simulating moist but not waterlogged soils. Anoxic conditions showed a protective role on viability at lower moisture contents (70% RH). Seed viability was negatively associated with hydrogen peroxide contents and correlated with antioxidant enzyme activities but not with membrane damage. Altogether, these results suggest negative roles for moist soils and anoxia in determining seed persistence in the field, but at higher moisture contents the negative effects of anaerobia diminished. The antioxidant system activation, even under unfavourable conditions, might recover seeds once all protective processes can operate, pointing out the plasticity of mechanisms involved in seed loss viability.

Keywords: **antioxidant enzymes, hydrogen peroxide, membrane damage, oxygen, *Ranunculus baudotii*, seed longevity**

Abbreviations: APX Ascorbate peroxidise**,** CAT Catalase**,** GPX Glutathione peroxidase**,** RH relative humidity**,** MC moisture content**,** ROS reactive oxygen species

Introduction

Seed persistence under field conditions is a key determinant of soil seed bank dynamics and knowledge of it is crucial to assess plant population dynamics and adaptation under both natural and agricultural ecosystems (Saatkamp *et al.,* 2014). Hence, effective management of plant populations, including restoration ecology, conservation of endangered species and weed eradication require knowledge of seed persistence (Bakker *et al.,* 1996; Panetta and Lawes 2005; Bossuyt and Honnay, 2008).

How long viable seeds persist in the field varies among species and populations, and depends on physical and physiological seed characteristics (inherent longevity) and how these are affected by biotic and abiotic factors (Long *et al.,* 2015). Whilst several studies found ecological correlates of abiotic factors with seed persistence in the field (Bekker *et al.,* 1998a; Abedi *et al.,* 2014) few studies have included soil water and temperature conditions (Bekker *et al.,* 1998b; Long *et al.,* 2009; Pakeman *et al.,* 2012). In addition, although seed persistence in the field has been shown to be predicted by laboratory-controlled ageing (Bekker *et al.,* 2003; Long *et al.,* 2008), most laboratory experiments were conducted under conditions not common under natural environments (Walters *et al.,* 2005). Hence, although it is well known that the longevity of seeds is mainly determined by seed moisture content and storage temperature, with life-span increasing with decreasing temperature and moisture content (Ellis and Roberts, 1980), the contribution of these factors on inherent longevity under near-natural conditions are not satisfactorily explored.

While temperature and moisture content are the two most important conditions affecting seed life-span, other abiotic factors also contribute to seed longevity in the field. For example, soils with high moisture levels are accompanied by low oxygen pressure and vice-versa. Oxygen might be expected to have both positive and negative effects on seed longevity. It tends to promote ageing at moisture contents below those at which respiration is possible and oxidative damage can accumulate but it delays ageing at higher moisture contents when respiration can occur and damage can be repaired (Ibrahim *et al.,* 1983; Roberts and Ellis, 1989; Vertucci and Leopold, 1984; Walters *et al.,* 2002). Hence, as under natural conditions seeds in the soil are metabolically active, increased water content of the soil could therefore be beneficial for seed survival but, on the other hand, effects of long periods of waterlogging could be negative when anoxic conditions occur (Bekker *et al.,* 1998b). The exact mechanisms that lead to the loss of seed viability have not been completely

elucidated. However, there is general consensus that oxidative and peroxidative processes play the primary roles in initiating damage (Bewley *et al.,* 2013). Particularly, reactive oxygen species (ROS) accumulation, lipid peroxidation and membrane damage are generally considered as the major contributors to seed deterioration (Hendry, 1993; Bailly, 2004; Kranner *et al.,* 2010). Damage due to free radicals and ROS is prevalent in dry seeds while at higher seed moisture contents, additional mechanisms of damage become possible mediated by enzyme activity (Walters *et al.,* 2002). Finally, seed deterioration is associated with a decreased activity of the detoxification system composed by a number of antioxidant enzymes which, in turn, changes in their activity may be considered good oxidative stress indicators (Bailly *et al.,* 2008).

The influence of temperature, moisture content and oxygen on seed longevity has been mostly examined to estimate viability of seed lots held in dry storage for long-term ex situ conservation of plant germplasm (Ellis and Roberts, 1980; Walters *et al.,* 2005; Groot *et al.,* 2015), hence, equilibrium relative humidity and temperature conditions, mostly used in a standard comparative longevity protocol (Newton et al., 2009), are respectively lower (60% RH) and higher (45 °C) than those usually present in natural environments. Indeed, very few studies have been undertaken on viability of seeds under conditions simulating a near-natural environment with a special focus on understanding seed persistence in the soil (Long *et al.,* 2009). Here, we aged seeds of *Ranunculus peltatus* Schrank subsp. *baudotii* (Godron) Meikle ex C.D.K. Cook (Ranunculaceae; hereafter *R. baudotii*) under three different relative humidities and under both aerobic and anoxic conditions, simulating the natural environment in the laboratory and assessed their viability, oxidative stress (hydrogen peroxide content) and changes in antioxidant defence system activity. As model species we selected *R. baudotii* because it is an hydrophyte constituting abundant soil seed bank (Rhazi *et al.,* 2001), germinating without requirement for a particular dormancy-breaking treatment (Carta *et al.,* 2012). In addition, as this species is widely distributed in temporary ponds of Southern Europe, we expect that the conclusions of our study could also be valuable, not only for other aquatic plants but also for terrestrial species growing in seasonally wet habitats.

Materials and methods

Seed material

Achenes of *Ranunculus baudotii* (hereafter referred to as seeds) were collected from approx. 100 plants of a population growing in the natural pond of Stagnone, Capraia Island, Italy (N 43.04, E 9.80) on 8 June 2016. Seeds were stored in the laboratory (approx. 50% relative humidity (RH) at 25±1°C) for 6 weeks before the starting the experiments.

Seed ageing treatments

A 2×3 factorial design was used to test the interactive effects of RH and oxygen on seed viability. Vials containing 60 seeds (for seed germination) and about 0.4 g (for membrane permeability, H_2O_2 contents, antioxidant enzymes) were pre-equilibrated above a non-saturated solution of LiCl held in a sealed box (Hay *et al.*, 2008) placed at 20±1 °C, creating an RH of 47±1% RH (395 gL^{-1} LiCl) for 3 days. At the end of this treatment seed moisture content was 10.97% ± 0.35. The ageing test started when these vials were transferred at 35±1 °C to sealed boxes with three relative humidities: 70±1% RH (250 gL^{-1} LiCl), 90±1% RH (100 gL^{-1} LiCl) and 97±1% RH (30 gL^{-1} LiCl) and two distinct gaseous environments (anaerobic conditions and aerobic conditions). Anaerobic conditions were achieved by adding ATCO oxygen absorbers sachets (Laboratoires Standa, Caen, France). On days 0, 2, 7, 9, 16, 30 and 50 of controlled ageing, vials were retrieved for moisture content, seed germination, membrane permeability, lipid peroxidation and antioxidant enzymes determination. A hysteresis effect when seeds were transferred to ageing cannot be ruled out (Hay and Timple, 2017), but if present it is of little concern to make comparisons across treatments because measurements of moisture content confirmed that the seeds were equilibrated at the desired RH.

These combinations were chosen for mimicking 6 distinct conditions the seeds may experience after dispersal in summer when the water table in the pond drops: the soil can have different moisture levels (dry, moist or wet) and the seeds can accumulate at the soil surface or be buried (limiting gas exchange). In addition, these moisture levels were chosen because they coincide with hydration levels II, III and IV, respectively, at which distinct deterioration and protection mechanisms are expected to be possible in seeds (Walters *et al.*, 2002; Bewley *et al.*, 2013).

Seed viability

Viability was assessed by a germination test in Petri dishes containing 1% distilled water-agar, incubated at a constant temperature of 10 °C with a 12h photoperiod (40-50 µmol $m^{-2}s^{-1}$). This condition was selected because it is optimal for germination of this species (Carta *et al.*, 2012). The experiment lasted for 50 days, during which germinated seeds were counted and removed every 5 days. Germination was defined as radicle emergence from the testa by at least 1 mm. At the end of the experiment cut tests determined the number of ungerminated but viable seeds with intact, white embryo and endosperm.

Determination of water content

Calculations of seed fresh weight, dry weight and moisture content (MC) were based on weights determined before and after oven drying of seed samples at 100 °C, until constant weight (Bass, 1979). Water content percentage was estimated on a fresh weight basis

Electrolytic conductivity method for membrane damage estimation

Membrane damage was estimated as in Spanò *et al.*, (2013) with minor modifications. Seeds (20 for each of the three repetitions) were incubated in deionised water and allowed stirring for 22h at 4 °C. The conductivity of the aqueous solution was measured with a Jeenway 4310 Conductivity Meter at 25 °C. Conductivity was also detected at 25 °C after boiling the test tube in a water bath for 2h. The extent of damage was calculated as percentage of membrane damage using the formula:

$$(C1 - Cw)/(C2 - Cw) \text{ x } 100$$

where C1 is electro-conductance value of samples at the first measurement, C2 is electro-conductance value after boiling and C_W is electro-conductance value of deionised water.

Extraction and determination of hydrogen peroxide and antioxidant enzymes

Seeds were milled (15 sec, 30 oscillations s^{-1}) in a steel-ball mill, cooled with dry ice. The powders were stored at -80 °C until their use for biochemical determinations. H_2O_2 content was determined according to Jana and Choudhuri (1982). The powder was extracted with phosphate buffer 50 mM pH 6.5 and the homogenate was centrifuged at 6000 *g* for 25 min. To determine the H_2O_2 content, 3 ml of extracted solution were mixed with 1 ml of 0.1% titanium chloride in 20% (v/v) H_2SO_4, after which the mixture was centrifuged at 6000 *g* for

15 min and the supernatant absorbance at 410 nm was read. The amount of H_2O_2 in the extracts was calculated from a standard curve and expressed as µmol g^{-1}FW.

To assess activity of the antioxidant enzymes, the extraction was made as in Spanò *et al.*, (2013), at 4 °C. The homogenate was then centrifuged at 15 000 *g* for 20 min. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). Enzyme activity was assayed from the decrease in absorbance at 290 nm (extinction coefficient 2.8 $mM^{-1}cm^{-1}$) as ascorbate was oxidised. Correction was made for the low, non-enzymatic oxidation of ascorbate by hydrogen peroxide (blank). Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined according to Navari-Izzo *et al.*, (1997) following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mm^{-1} cm^{-1}). Catalase (CAT; EC 1.11.1.6) activity was determined as described by Aebi (1984). Specific activity was calculated from the 39.4 mM^{-1} cm^{-1} extinction coefficient. A blank containing only the enzymatic solution was made. All enzymatic activities were determined at 25 °C and expressed as U mg^{-1} protein. Protein measurement was performed according to Bradford (1976), using BSA as standard.

Statistical analysis

Seed viability was modelled by fitting the viability equation (Ellis and Roberts 1980):

$$v = K_i - (p/\sigma),$$

where v is the viability (in normal equivalent deviates, NED) of the seed lot after *p* days, K_i is the initial viability (NED) of the seed lot, and σ is the time (d) for viability to fall by 1 NED (i.e. the standard deviation of the normal distribution of seed deaths over time). In fitting the Ellis and Roberts (1980) viability equations, K_i is usually constrained to a single estimate for all ageing experiments on a particular seed lot. Hence, a generalized linear models (GLM) with binomial error and probit link function was fitted to the survival data (numbers of seeds germinating, number of seeds sown) in R (R Development Core Team, 2016) with ageing time as explanatory variable, and ageing conditions as fixed factors. The effect of dropping intercept was also considered, using an *F*-test to assess significance. The effects of ageing treatments on membrane damage, on H_2O_2 contents and on antioxidant enzymes contents were assessed by means of linear models while their correspondences with mean final germination (viability) was modelled using binomial GLMs.

Results

Seed viability

When fitting the probit GLM the main effect of oxygen on viability was significantly positive ($P = 0.01$). However, the interaction of 70% RH (~12.5% MC, Table 1) with oxygen was not significant ($P > 0.05$), i.e. at 70% RH the differences in viability were not significantly different regardless whether the seeds were aged in aerobic or anaerobic conditions. On the contrary, whilst the main effect of relative humidity was not significant, all slope terms were highly significant ($P < 0.001$) and indeed seed viability declined as the period of experimental ageing increased, for all treatments (Fig. 1) with a significant variation in the estimate for deterioration rate σ (7.3–22.7 d) and thus in the time taken for viability to fall to 50% ($p50$; 10.1–69.2 d) between ageing treatments (Table 1). Dropping the intercept (K_i) term from the model resulted in a significant increase in residual deviance ($P < 0.001$) and hence the final model fitted is that showing a single estimate of K_i for all ageing experiments (Table 1).

Seed samples aged under aerobic conditions showed a slower rate of viability loss and survived longer compared with seeds aged at the corresponding relative humidity under anaerobic condition, except the loss of viability at 70% RH when deterioration rate was a little faster ($\sigma = 18.18$ d) in the presence of oxygen compared with the sample aged under anaerobic condition ($\sigma = 18.52$ d; Table 1). Nevertheless, as discussed above, the interactive effect of 70% RH with oxygen was not significant. Despite the overall beneficial effect of oxygen, the longevity at 90% RH (~15% MC, Table 1) showed a marked reduction ($\sigma =$ 12.35 d) compared to 97% ($\sigma = 22.73$ d). On the contrary, under anaerobic condition at 97% RH (~23% MC, Table 1), seed viability declined much more and faster than at 70% RH (Fig. 1).

Membrane permeability

Overall, no significant ($P > 0.05$) changes of membrane damage during the time of treatments were detected nor was there a significant association of damage with H_2O_2; however, membrane damage generally significantly increased under aerobic conditions ($P < 0.001$) and

with increasing moisture content ($P < 0.05$). At 70% RH the damage was negligible under both aerobic and anaerobic conditions.

Membrane damage was not generally significantly ($P > 0.05$) associated with H_2O_2 concentration nor with seed viability. This was apparently caused by the opposite association of damage with viability at 97% and 90% RH: significantly ($P < 0.001$) negative under aerobic conditions and positive ($P > 0.05$) under anaerobic conditions.

Hydrogen peroxide

Overall, there were significant positive effects of oxygen and duration of ageing on H_2O_2 contents (Table 2). However, we could not find a general significant effect of RH on H_2O_2 contents. Nevertheless, the highest H_2O_2 concentrations were detected at 97% RH, especially under aerobic condition (Fig. 2). In addition, hydrogen peroxide concentrations recorded at 70% and 90% RH were slightly lower under aerobic than under anaerobic conditions. An overall negative association ($P < 0.001$) of H_2O_2 contents with viability was found and this effect on seed viability was similar in all ageing treatment (Table 3) except for 70% RH under anaerobic condition which viability was unaffected by H_2O_2. The estimate coefficients are however larger at 90% and 97% RH anaerobic and 70% RH aerobic suggesting that the negative effect of H_2O_2 on seed viability is particularly strong under such conditions.

Antioxidant enzymes

While H_2O_2 content and oxygen were not generally associated with APX, the decrease of the activity of this enzyme depended on RH, with an overall significant reduction at 70% and 97% RH (Table 4, Fig. 2). Under anaerobic conditions, the decrease of APX activity was particularly strong at 97% while under aerobic conditions a significant reduction was detected only at 90% RH. No general relation between seed viability and APX activity was found; however, by considering the aerobic treatments alone, a significant ($P < 0.001$) positive association was found. Nevertheless, considering all treatments separately, significant positive relations of APX with viability were recorded for all conditions, especially for 97% RH anaerobic (Table 5).

Hydrogen peroxide and oxygen were not significantly associated with GPX activity while RH levels significantly explain the variation in GPX activity detected. Indeed, GPX is generally favoured by higher RH; in addition, under aerobic conditions a significant increase

was detectable mostly at 90% RH while under anaerobic conditions no significant variation was found. Overall, there was a significant ($P < 0.001$) negative association between GPX activity and viability but this effect largely varied among ageing treatments (Table 5). Indeed, besides a strong negative effect at 90% RH aerobic, a non-significant effect was present at 90% RH anaerobic whereas the contrary was evident for 70% RH. Finally, moderate negative effects were evident for both aerobic and anaerobic conditions at 97% RH.

Both hydrogen peroxide and oxygen were positively associated with CAT activity that decreased, depending on RH in particular, at 97% and 70% RH (Table 4). Under anaerobic conditions, the decrease in activity was higher at 97% and 70% RH while under aerobic conditions the activity of CAT was significantly reduced at 70% RH only. We found no overall significant association between CAT activity and viability over the duration of ageing but when results were analysed separately among treatments, positive significant effects were shown for all treatments except at 90% RH in absence of oxygen (Table 5).

Discussion

The rate of ageing is known to be determined by the species, the seed moisture content and the temperature and duration of the ageing process (Ellis and Roberts, 1980; Bekker *et al.*, 2003; Probert *et al.*, 2009) but also by the gaseous environment (Ibrahim and Roberts, 1983; Roberts and Ellis, 1989). In our experimental design we focused on the relative influence of water and oxygen availability and used a single temperature condition because this is the temperature the seeds may experience after dispersal in a Mediterranean climate. As seed viability loss clearly depended on ageing conditions simulating the natural environment in summer (Table 1), our ageing treatments in the laboratory afforded insights into the effects of soil moisture and oxygen on the persistence of seeds in the field before germination might take place in autumn (Carta *et al.*, 2012). The physiological causes of ageing have been largely studied but they are undoubtedly complex and the variation of patterns observed at different moisture contents pointed out controversial roles of the oxidative processes in seed ageing (Bailly *et al.*, 2008). Similarly, whilst antioxidant enzyme activities were generally positively associated with seed viability (Table 5), the single enzyme activity and its supposed beneficial effects varied among treatments, suggesting possible reorientation of the enzymatic antioxidant defence system, depending on ageing condition.

Membrane damage is one of the main alterations occurring during storage (Fotouo-M *et al.,* 2015) and the electrical conductivity (EC) test, indicative of membrane injury, is often used as an effective method to assess seed viability (Fessel *et al.,* 2006). Indeed, in our study EC-increase under aerobic conditions and with increasing hydrogen peroxide concentrations at high RH is consistent with an oxidative damage to membranes. Unlike the negative correlation often detected between viability and membrane damage (Shereena and Salim, 2006; Singh and Richa, 2016), the lack of association between EC and viability recorded in our seeds, suggests that membrane damage is not the main cause of loss of germination ability, at least under anaerobic conditions. On the other hand, the strong negative association between hydrogen peroxide and viability (Table 3) seems to indicate that membranes are not the main target of oxidative damage and other damaging events could occur to stored oils (*R. baudotii* seeds have about 15% of oil content, Guil-Guerrero *et al.,* 2001) or to the non-lipid cellular fraction (Kibinza *et al.,* 2006; Morsher *et al.,* 2015).

Hydrogen peroxide is a key ROS that being rather stable, is able to cross cell membranes and can act as a signalling molecule, able to activate antioxidant protective response. The increase in H_2O_2 content detected under anaerobic conditions (Fig. 2) confirms the possibility of accumulation of this ROS also in the absence of oxygen or in hypoxic conditions (Paradiso *et al.,* 2016). Noteworthy, the highest contents of hydrogen peroxide were recorded in aerobic conditions with high RH where, however, viability remained high. This could be due to the significant positive effect of antioxidant enzymes at 97% RH, highlighting the importance of an adequate metabolic recovery possible only at the highest moisture contents (Bewley *et al.,* 2013). The anoxic conditions seem to play a protective role in viability only at 70% RH, confirming previous data (Ellis and Hong, 2007) on *Phleum pratense* and *Sesamum indicum,* showing a better longevity in hermetic storage at low RH values. In contrast, oxygenic conditions were necessary for a longer-lasting germination ability at both 90 and 97% RH (Table 1; Fig. 1). Moreover, previous findings reported that seed viability under anaerobic conditions at very high moisture contents cannot be maintained for more than a few days (Ibrahim *et al.,* 1983; Roberts and Ellis, 1989). However, in our study survival was lowest at 90% RH while at 97% RH anaerobic seed survival was slightly higher suggesting that protective mechanisms may be effective even in absence of oxygen. Indeed, we found a strong positive association of the antioxidant system (particularly APX) with seed viability at 97% RH anaerobic (Table 5). The positive action of antioxidant enzymes in seeds with moisture content higher than the lower limit for respiration is consistent with an increased ROS production due to electron transport chain (ETC) disorganization under low oxygen conditions

(Bewley *et al.*, 2013). Accordingly, in our study the difference between aerobic and anaerobic ageing was mostly marked at very high moisture content (97% RH). Instead, at 90% RH, simulating moist, non-waterlogged soils, survival was lowest under both aerobic and anaerobic conditions. It should be noted that this level of humidity approximately coincides with the minimum limit for respiration (Bewley *et al.*, 2013), that is to say that at 90% RH even under aerobic condition respiratory activity may be inefficient but sufficient water is available allowing most damaging reactions (e.g. chemical, ROS, enzymatic and some metabolic) to occur. Indeed, the ability of seeds to maintain a good viability also depends on its capacity to sustain a good antioxidant machinery (Kumar *et al.*, 2015).

Ascorbate peroxidase and catalase are two H_2O_2-detoxifying enzymes, playing an important role in the control of this reactive molecule (Anjum *et al.*, 2016). GPX, besides scavenging H_2O_2, can detoxify lipid hydroperoxides and other reactive molecules under several stress conditions (Bela *et al.*, 2015). There was a general decrease in APX activity with the time of treatment (albeit not significant, Table 4) with a trend that was opposite to that of the content of hydrogen peroxide. The activity of CAT was lower under anaerobic conditions (Table 4), and so the high concentrations of hydrogen peroxide in the absence of oxygen could also be derived from the low activity of these H_2O_2-scavenging enzyme. Nevertheless, we found a strong positive association of APX with seed viability and significant positive effects of CAT for all treatments, except 90% RH anaerobic (Table 5). We also found an overall increase of GPX with time of ageing under aerobic condition (Fig. 2) but the effect of this enzyme on viability was not clear; its increase may simply be an indication of stress (Hossain *et al.*, 2015) and might be linked with detoxification of lipid peroxidation products which may be formed due to the activity of active oxygen species (Eshdat *et al.*, 1997). Several studies have demonstrated that seed ageing is associated with a loss of antioxidant enzyme activity (Bailly, 2004) and this tendency was also found in our study. On the other side, we also found good correlations of seed viability with enzyme activity. Nevertheless, if only dead seeds lose a given component antioxidant activity, it is not easy to distinguish cause and effect (Bewley *et al.*, 2013). Hence, we regard with caution the resulted associations of enzyme activities with seed viability.

Studies investigating the influence of the water regime on the species composition of the soil seed bank under natural conditions present contrasting results with seeds of species from wet habitats that may better tolerate anoxic conditions (Bekker *et al.*, 1998a; Murdoch and Ellis, 2000). Wetland species accumulate dense seed banks in waterlogged soils and presumably such species have metabolic adaptations that permit them to survive also in the

absence of oxygen (Bekker *et al.*, 1998b). Transferring our results to field conditions, the capacity for *R. baudotii* seeds to survive better at 97% RH anaerobic compared to 90% RH anaerobic appears to be consistent with this. Indeed, numerous records of seed survival in the soil (Bonis *et al.*, 1995; Rhazi *et al.*, 2001; A. Carta unpublished data) provide evidence that *R. baudotii* seeds may remain viable for years under conditions where they are fully hydrated for considerable periods. Such observations are compatible with our laboratory experiments also considering that for a large part of the year the soil temperature is significantly lower than the one used in our experiment. Only during summer the temperature and humidity levels may be close those used in our experiments (Casas and Ninot, 2007).

In the field, seeds in strongly seasonal climates can experience daily and seasonal fluctuations in temperature and moisture, particularly in the upper centimetres of soil (Benvenuti *et al.*, 2001; Saatkamp *et al.*, 2011). It is not necessary for seeds to be fully hydrated continuously in order to maintain viability for extended periods: intermittent hydration is adequate, assuming that the dry periods are not too long (Long *et al.*, 2011). Similarly, future studies should explore the role of intermittent aeration in reinstalling the antioxidant capacity after the seeds experienced short period of anoxic conditions. Altogether, these results suggest a negative role for moist soils in determining the persistence of seeds in the field. The ability to activate the antioxidant system even under these unfavourable conditions might however help seeds to recover once they are in wet soils, when most protective processes can fully operate. These data underscore the plasticity of the mechanisms involved in seed loss viability and may in part explain the controversy about the role of the oxidative processes in seed ageing.

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Conflicts of interest

None

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Table 1. Survival curve parameters for each ageing treatment determined using a generalized linear model with binomial error and probit link function. Ageing conditions (relative humidity and oxygen) were included as factors. Estimates of K_i could be constrained to a single value for the different ageing treatments. K_i, (± SE) initial viability [normal equivalent deviates (NED)] of the seed lot; σ, time (d) for viability to fall by 1 NED; p_{50} (± SE), time (d) for viability to fall to 50%. MC, mean (± SE) moisture content during experimental treatments.

Treatment	***MC***	**K_i (NED)**	**σ^{-1} (d^{-1})**	**σ (d)**	**p_{50} (d)**
70% RH aerobic	12.23 ± 0.24	1.31 ± 0.03	0.055 ± 0.002	18.18	29.288 ± 1.675
70% RH anaerobic	12.75 ± 0.53		0.054 ± 0.002	18.52	31.198 ± 1.752
90% RH aerobic	14.65 ± 0.63		0.081 ± 0.004	12.35	16.249 ± 0.913
90% RH anaerobic	14.58 ± 0.42		0.137 ± 0.011	7.30	10.153 ± 0.480
97% RH aerobic	23.38 ± 1.36		0.044 ± 0.002	22.73	69.253 ± 8.820
97% RH anaerobic	21.63 ± 0.94		0.068 ± 0.003	14.71	20.209 ± 0.988

Table 2. Simple linear regressions results for the effect of ageing duration and ageing conditions on hydrogen peroxide concentration (fitted separately). Ageing duration was included as continuous variable, oxygen and relative humidity were included as factors. Significant results are in bold.

	Estimate	**SD**	**t**	***P***
Ageing duration	0.035	0.006	5.499	**0.0000**
Oxygen	0.308	0.134	2.304	**0.0230**
70% RH	0.589	0.384	1.532	0.1282
90% RH	0.573	0.384	1.491	0.1387
97% RH	0.926	0.384	2.408	**0.0176**

Table 3. Generalised linear models (GLMs , binomial error, logit link) results for the effect of hydrogen peroxide concentration on seed viability (fitted separately for each treatment). H_2O_2 concentration was included as continuous variables. Significant results are in bold.

	Estimate	**SD**	**t**	***P***
70% RH aerobic	-1.627	0.258	-6.302	**0.0000**
70% RH anaerobic	-0.409	0.293	-1.395	0.1629
90% RH aerobic	-0.913	0.307	-2.976	**0.0029**
90% RH anaerobic	-2.306	0.199	-11.600	**0.0000**
97% RH aerobic	-0.417	0.135	-3.090	**0.0020**
97% RH anaerobic	-3.382	0.337	-10.040	**0.0000**

Table 4. Simple linear regressions results for the effect of ageing duration, ageing conditions and hydrogen peroxide concentration on antioxidant enzymes activity (fitted separately). Ageing duration and H_2O_2 concentration were included as continuous variables, oxygen and relative humidity were included as factors. Significant results are in bold.

		Estimate	**SD**	**t**	***P***
APX	Ageing duration	-0.001	0.001	-1.758	0.0813
	Oxygen	-0.008	0.013	-0.619	0.5370
	70% RH	-0.086	0.035	-2.443	**0.0160**
	90% RH	-0.058	0.035	-1.633	0.1050
	97% RH	-0.106	0.035	-3.004	**0.0032**
	H_2O_2	-0.008	0.008	-1.002	0.3180
GPX	Ageing duration	0.002	0.002	1.064	0.2900
	Oxygen	0.057	0.035	1.630	0.1060
	70% RH	0.221	0.090	2.444	**0.0160**
	90% RH	0.410	0.090	4.536	**0.0000**
	97% RH	0.331	0.090	3.657	**0.0004**
	H_2O_2	0.014	0.023	0.577	0.5650
CAT	Ageing duration	-0.010	0.006	-1.729	0.0863
	Oxygen	0.548	0.102	5.360	**0.0000**
	70% RH	-1.573	0.247	-6.359	**0.0000**
	90% RH	-0.659	0.247	-2.663	**0.0088**
	97% RH	-1.224	0.247	-4.946	**0.0000**
	H_2O_2	0.160	0.075	2.144	**0.0340**

Table 5. Generalised linear models (GLMs , binomial error, logit link) results for the effect of antioxidant enzymes activity on seed viability (fitted separately for each treatment). Enzymes concentration were included as continuous variables. Significant results are in bold.

		Estimate	**SD**	**t**	***P***
APX	70% RH aerobic	5.971	1.899	3.145	**0.0017**
	70% RH anaerobic	5.681	1.523	3.730	**0.0002**
	90% RH aerobic	7.139	1.639	4.356	**0.0000**
	90% RH anaerobic	5.253	2.380	2.208	**0.0273**
	97% RH aerobic	6.240	3.164	1.972	**0.0486**
	97% RH anaerobic	15.389	3.079	4.998	**0.0000**
GPX	70% RH aerobic	0.217	0.523	0.415	0.6780
	70% RH anaerobic	-3.594	0.757	-4.751	**0.0000**
	90% RH aerobic	-4.436	0.555	-7.990	**0.0000**
	90% RH anaerobic	-0.208	0.540	-0.385	0.7000
	97% RH aerobic	-1.526	0.614	-2.487	**0.0129**
	97% RH anaerobic	-1.220	0.518	-2.353	**0.0186**
CAT	70% RH aerobic	0.412	0.166	2.479	**0.0132**
	70% RH anaerobic	0.645	0.185	3.490	**0.0005**
	90% RH aerobic	3.776	0.396	9.543	**0.0000**
	90% RH anaerobic	0.349	0.223	1.561	0.1190
	97% RH aerobic	1.113	0.394	2.824	**0.0048**
	97% RH anaerobic	0.971	0.171	5.682	**0.0000**

Legends to figures

Figure 1. Relationships between *p*50, time (d) for viability to fall to 50% and experimental relative humidity (RH) for seeds of *Ranunculus baudotii* under either aerobic (red line) or anaerobic (black line) conditions.

Figure 2. Antioxidant enzyme activities (±SE) and H_2O_2 content (as indicated) for treatments under aerobic conditions (open symbols) and for those under anaerobic conditions (closed symbols) at 35 °C and 70% (squares), 90% (triangles) and 97% RH (circles).

Supplementary material

Figure S1. Survival curves fitted using a generalized linear model with binomial error and probit link function for treatments under aerobic conditions (open symbols) and for those under anaerobic conditions (closed symbols) at 35 °C and 70% (squares), 90% (triangles) and 97% RH (circles).

Figure 1

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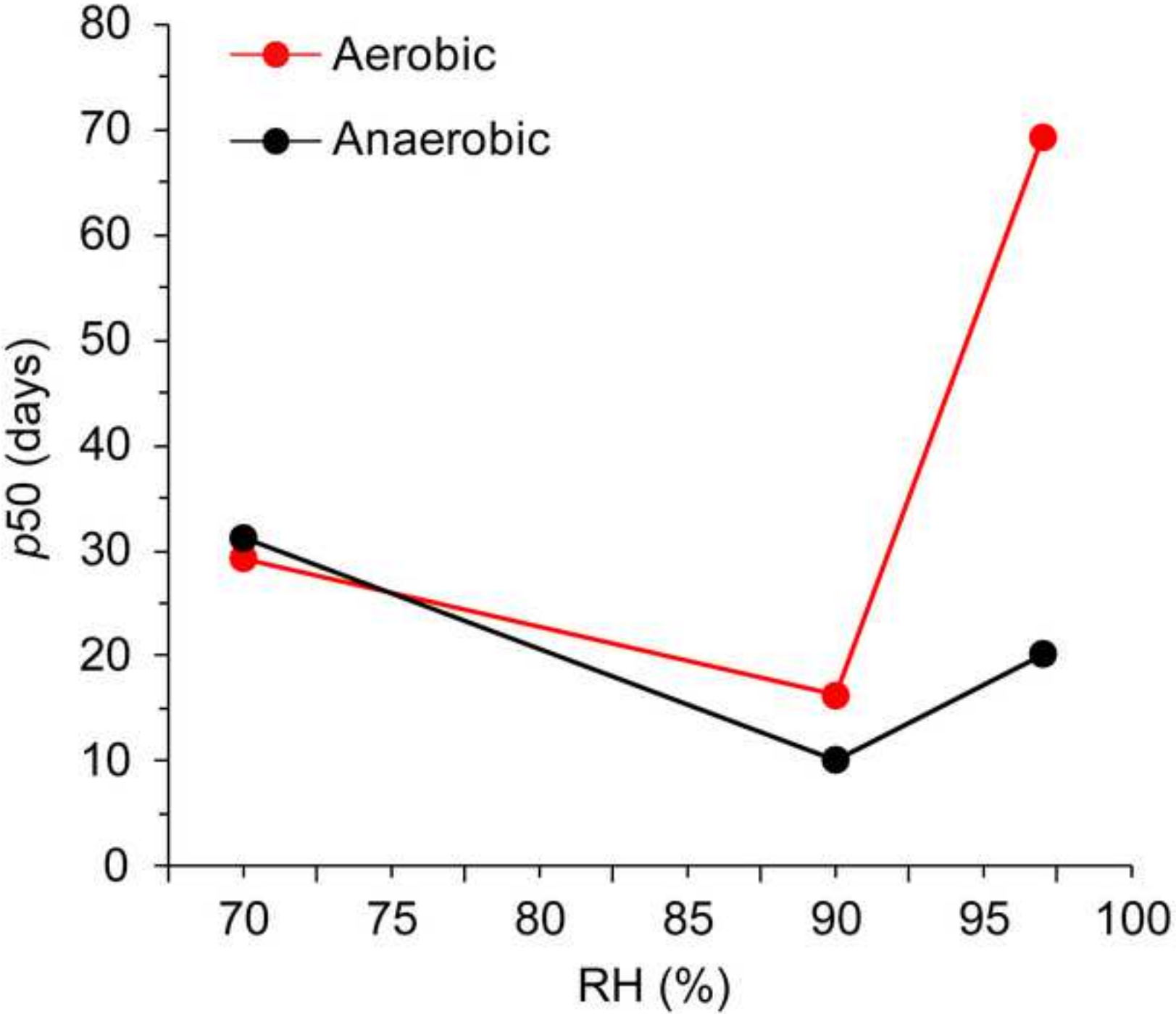

Figure 2

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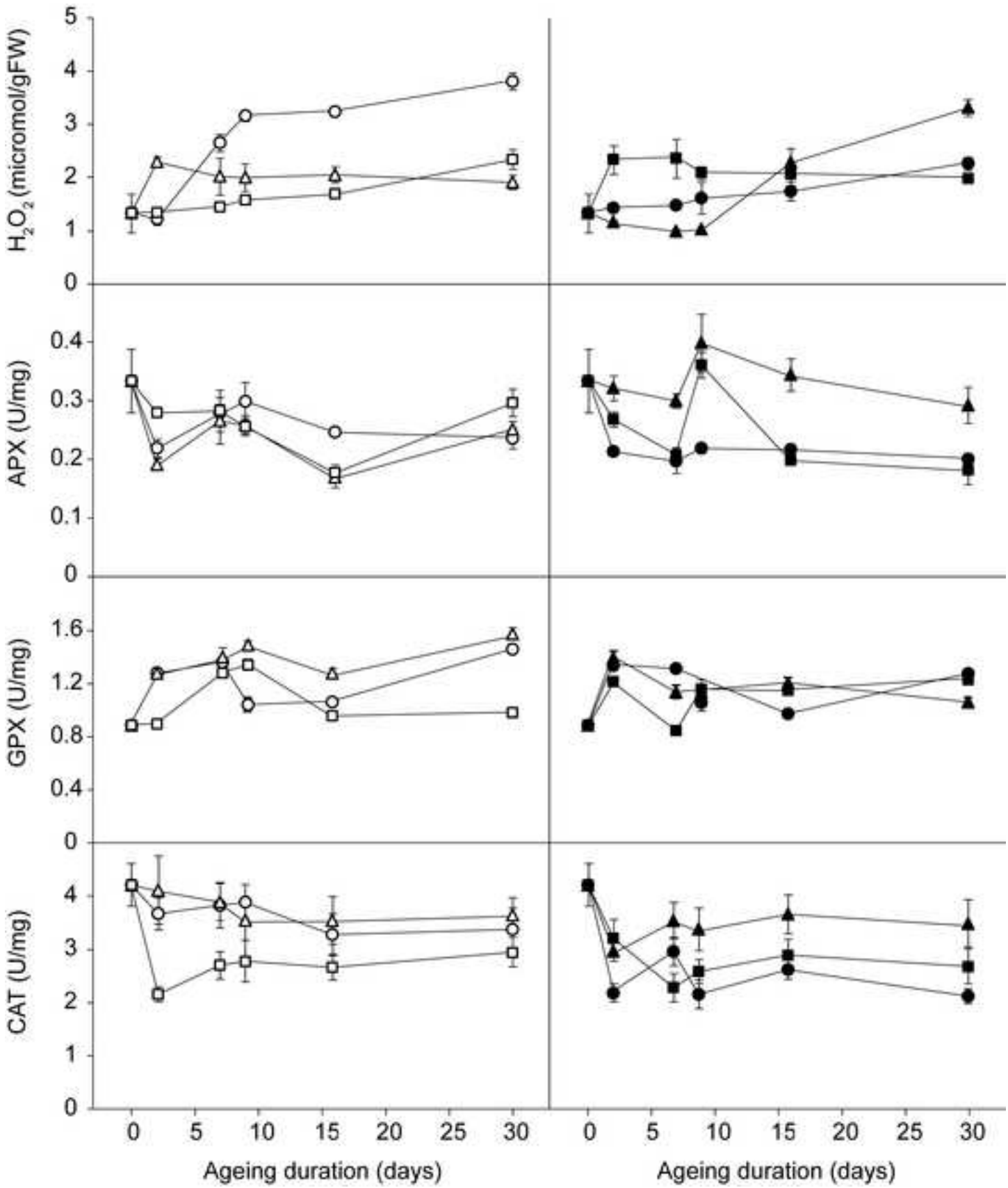

Figure S1

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