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DNA profiling, telomere analysis and antioxidant properties as tools for monitoring *ex situ* seed longevity

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• *Background and Aims* The germination test currently represents the most used method to assess seed viability in germplasm banks, despite the difficulties caused by the occurrence of seed dormancy. Furthermore, seed longevity can vary considerably across species and populations from different environments, and studies related to the eco-physiological processes underlying such variations are still limited in their depth. The aim of the present work was the identification of reliable molecular markers that might help in monitoring seed deterioration.

• *Methods* Dry seeds were subjected to artificial ageing and collected at different time points for molecular/biochemical analyses. DNA damage was measured using the RAPD (random amplified polymorphic DNA) approach while the seed antioxidant profile was obtained using both the DPPH (1,1-diphenyl, 2-picrylhydrazyl) assay and the Folin–Ciocalteu reagent method. Electron paramagnetic resonance (EPR) provided profiles of free radicals. Quantitative real-time polymerase chain reaction (QRT-PCR) was used to assess the expression profiles of the antioxidant genes *MT2* (type 2 metallothionein) and *SOD* (superoxide dismutase). A modified QRT-PCR protocol was used to determine telomere length.

• *Key Results* The RAPD profiles highlighted different capacities of the two *Silene* species to overcome DNA damage induced by artificial ageing. The antioxidant profiles of dry and rehydrated seeds revealed that the high-altitude taxon *Silene acaulis* was characterized by a lower antioxidant specific activity. Significant upregulation of the *MT2* and *SOD* genes was observed only in the rehydrated seeds of the low-altitude species. Rehydration resulted in telomere lengthening in both *Silene* species.

• *Conclusions* Different seed viability markers have been selected for plant species showing inherent variation of seed longevity. RAPD analysis, quantification of redox activity of non-enzymatic antioxidant compounds and gene expression profiling provide deeper insights to study seed viability during storage. Telomere lengthening is a promising tool to discriminate between short- and long-lived species.

Key words: Antioxidant potential, *ex situ* seed longevity, QRT-PCR, RAPD, *Silene vulgaris* subsp. *vulgaris*, *Silene acaulis* subsp. *acaulis*, telomere.

INTRODUCTION

Seed longevity is a relevant trait from an ecological perspective, playing a crucial role in safeguarding vulnerable plant species which are preserved *ex situ* in seed banks (Li and Pritchard, 2009; Probert *et al.*, 2009). It is generally acknowledged that seeds stored for prolonged periods are subjected to severe oxidative damage, caused by the progressive accumulation of reactive oxygen species (ROS) and that loss of seed viability and reduced germination represent the undesired consequences of ageing (Kranner *et al.,* 2010). Significant factors in seed longevity are the level of DNA damage and the DNA repair response, the amount of non-enzymatic antioxidants and activity of ROS-scavenging enzymes (Rajjou and Debeaujon, 2008; Ventura *et al.*, 2012).

In order to preserve the high seed viability at the pre-emergence step, both the DNA repair functions and the overall antioxidant activities must be kept at an appropriate level in the embryo. Different DNA repair pathways are activated during the early phase of seed imbibition, as recently demonstrated by gene profiling studies (Macovei *et al.*, 2010, 2011*a*, *b*; Balestrazzi *et al.*, 2011*a*, 2012). The ability to carry out ROS scavenging, expressed as the seed antioxidant potential, is a critical requirement to withstand stress and improve germination (Liu *et al.*, 2007). The cell antioxidant systems prevent ROS attack but, when ROS production exceeds the capacity of the antioxidant machinery, oxidative injury takes place.

Factors such as temperature and humidity are positively correlated with seed ageing and they must be strictly controlled during seed manipulation for long-term conservation in seed banks (Walters *et al.*, 2005). To date, germination tests represent the most reliable method to assess seed viability (Smith *et al.*, 2003) although it is a time-consuming and labour-intensive operation. For this reason, novel low-cost and equally reliable methods are required, which might speed up the seed viability analysis. Molecular and biochemical markers of seed ageing might be used for these purposes. A deeper understanding of the complex network of molecular events which control seed

longevity is, however, required in order to select appropriate markers providing information on deterioration and germination potential of seed stocks collected for bank storage.

The effects of ageing on DNA integrity can be investigated using the random amplification of polymorphic DNA (RAPD), a simple and economical procedure for detecting DNA polymorphisms. Polymorphic RAPD profiles were detected in soybean (*Glycine max*) seeds of different ages (Bednarek *et al.*, 1998), while no differences were observed between seeds subjected to artificial and natural ageing (Marcos-Filho and McDonald, 1998). More recently, Vijay *et al.* (2009) reported changes in DNA profiles of soybean and safflower (*Carthamus tinctorius*) seeds exposed to natural and accelerated ageing, respectively.

An intriguing aspect of ageing is related to the role played by telomeres, nucleoprotein structures located at the end of chromosomes, which are essential to preserve genome integrity. As in animal cells, plant telomere DNA is a dynamic structure subjected to shortening during differentiation and ageing (Kilian *et al.*, 1995). Telomere monitoring has been suggested as a reliable marker of seed ageing. Bucholc and Buchowicz (1992) demonstrated significant differences in the length of telomere sequence between fresh and stored wheat (*Triticum aestivum*) seeds, despite their similarity in terms of germination percentage.

Several reports have highlighted the relevance of seed antioxidant ability as an indicator of longevity. According to Talai and Sen-Mandi (2010), the total antioxidant potential in fresh harvested seeds of different varieties is under direct genetic control, whereas environmental factors might act in aged seeds. Methodologies based on the use of the 1,1-diphenyl, 2-picrylhydrazyl (DPPH) radical are frequently utilized to assess the radical-scavenging activity of phenolic compounds (Hasan *et al.*, 2009). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides.

Antioxidant enzyme activities are also essential to protect the cellular components against oxidative injury. Superoxide dismutase (SOD) catalyses the dismutation of superoxide radicals to hydrogen peroxide and oxygen (Raychadhuri and Deng, 2000). The requirement for SOD activity, which plays a critical role in maintaining ROS at non-toxic levels during germination, has been reported in several plant species (Wojtyla *et al.*, 2006), and in a recent work Lee *et al.* (2010) demonstrated that the overexpression of the *SOD* gene in *Nicotiana tabacum* resulted in protective effects against seed deterioration during ageing.

Metallothioneins (MTs) have recently been proposed to act as ROS scavengers and signal molecules outside and inside the nucleus, highlighting their possible interaction with the DNA repair machinery (Wang *et al.*, 2010; Balestrazzi *et al.*, 2011*b*). To date, there is only indirect evidence of the putative protective role played by MTs in the nucleus. Balestrazzi *et al.* (2009) demonstrated that expression of the *PsMT$_{A1}$* gene, encoding an MT-like protein from *Pisum sativum*, confers protection against oxidative stress in the nucleus, reducing the level of oxidative DNA damage. As for the possible role of MTs in seed physiology, the seed-specific type 4 MTs possess a high capacity to bind Zn ions, compared with other isoforms. In *Arabidopsis thaliana*, the *MT4a* and *MT4b* mRNAs are accumulated during late embryogenesis and disappear rapidly following seed imbibition (Kranner and Colville, 2010). Metallothioneins have also been investigated in the genus *Silene*, and copper-tolerant populations of *Silene vulgaris* have significantly higher levels of the *SvMT2b* transcript, compared with copper-sensitive populations (Mengoni *et al.*, 2001).

Among seeds of different species stored under identical conditions there may be a wide inherent variation of seed longevity (Priestley *et al.*, 1985). Such variation has been reported to be related to taxonomy, seed structure and climate at the geographic origin of the species (Probert *et al.*, 2009; Mondoni *et al.*, 2012*a*, *b*). Moreover, Mondoni *et al.* (2011) found that alpine plants have short-lived seeds in storage compared with those from lowland populations/related taxa, highlighting a significant concern for the successful *ex situ* conservation of these species. The reduced longevity of seeds of alpine plants was suggested to be caused by low selection pressure for seed resistance to ageing and/or damage occurring during seed development, due to the cool wet conditions of the alpine climate. More detailed studies are therefore required to better understand the processes responsible for the different degree of seed viability in species and populations originating from different environments.

The aim of this investigation was the identification of reliable markers of seed deterioration. The response to DNA damage induced by artificial ageing was compared in seeds of *S. vulgaris* and *S. acaulis* inhabiting low- and high-altitude locations of Northern Italy, respectively. A more in-depth investigation which included ROS accumulation profiles, antioxidant capacity and telomere length was carried out, focusing mainly on dry seeds and seeds subjected to rehydration. Previous investigations have demonstrated that these species differ in seed longevity (Mondoni *et al.*, 2011), making them useful candidates to assess novel markers of seed deterioration.

MATERIALS AND METHODS

Seed material

Seeds of *Silene vulgaris* subsp. *vulgaris* (Moench) Garke and *Silene acaulis* (L.) Jacq. subsp. *acaulis* (http://ww2.bgbm.org/EuroPlusMed/PTaxonDetails.asp?NameId=104374&PTRefFk=7200000) were collected at the point of natural dispersal from one lowland (44°33′N, 12°16′; 0 m a.s.l.) and one alpine location (46°2′N, 9°31′; 2213 m a.s.l.), respectively, in Northern Italy, between May and September 2010. After collection, seeds were held at the Lombardy Seed Bank (Rossi and Mondoni, 2006) under international seed bank standard conditions of − 20 °C after drying at 15 % RH (relative humidity), 15 °C (FAO/IPGRI, 1994) until use.

Artificial ageing and germination tests

Due to logistical and time constraints in comparing storage life span under seed bank conditions, seed longevity was determined using a standard rapid ageing protocol (Probert *et al.*, 2009), to raise the moisture content of the seeds prior to ageing. In order to minimize the subsequent adjustment of

moisture content when samples were transferred to the ageing conditions, ten samples of 50 seeds each were rehydrated at 47 % RH at 20 °C in open glass vials or Petri dishes. The vials/dishes were placed over a non-saturated solution of LiCl (VWR International, Milan, Italy) in distilled water held in a sealed $300 \times 300 \times 130$ mm electrical enclosure box (Ensto UK Ltd, Southampton, UK). At the end of the rehydration period (14 d), seed equilibrium relative humidity (eRH) was checked using a sample of the equilibrating seeds. The eRH was evaluated using a water activity measuring instrument which comprised a hygrometer sensor housed in an AW-DI0 water activity probe, in conjunction with a HygroPalm 3 display unit (Simens VDO, Milan, Italy). Once the test species was judged to have reached equilibrium, samples were transferred to a second electrical enclosure box, over a non-saturated solution of LiCl at 60 % RH placed in a compact incubator (Binder FD53, VWR International) without light at 45 ± 2 °C. One sample (50 seeds for each seed lot) was removed after 1, 2, 5, 9, 20, 30, 50, 75, 100, 120 and 150 d and used for germination tests. Seeds were sown on 1 % distilled water agar held in 90 mm diameter Petri dishes and placed in an LMS 250A cooled incubator (LMS Ltd, Sevenoaks, UK) at a temperature regime previously found to be optimal for germination of that accession. Plates were checked weekly for germination and seeds scored as germinated once the radicle had reached 2 mm. At the completion of each germination test, ungerminated seeds were cut-tested to confirm that they were not viable.

Probit analysis

Probit analysis was carried out on the data obtained from germination tests using GenStat Release 11·1 (VSN International Ltd, Oxford, UK) to estimate the time for viability to fall to 50 % (*p*50) 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 in storage, 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). Analysis of residual deviance (variance ratio test following the *F*-distribution) was used to test for significance when constraining survival curve data for multiple seed lots to common estimates for K_i and/or σ.

Electron paramagnetic resonance (EPR)

Samples (15 seeds) of *S. vulgaris* and *S. acaulis* (8·5 and 4·3 mg, respectively) were inserted in quartz tubes and the EPR spectra were recorded with a Bruker EMX/12 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) operating in X-band, at room temperature and under RH conditions dictated by the silica gel used as a desiccant. Spectra were recorded with the same instrumental settings, under conditions far from saturation and performing accumulations for low intensity signals. Power saturation curves were obtained by plotting the EPR signal intensity against the square root of microwave power applied to the sample. The comparison between the two species was done by considering the areas of the spectra recorded at 0·01 mW, well away from saturation conditions. The areas of the EPR signals were calculated by double integration and normalized to seed weight and number of accumulations. The *g*-values were calculated with reference to a standard of Cr^{3+}/MgO with $g = 1.9797$.

DNA extraction and RAPD analysis

Genomic DNA was extracted and purified from dried, rehydrated and artificially aged *Silene* seeds using the NucleoSpin® Plant II kit (Macherey-Nagel, Duren, Germany), according to the manufacturer's instructions. For each sample, 100 mg aliquots (corresponding to approx. 100 seeds) were used. RAPD analysis was performed as follows. Twenty Operon primers (Eurofins MWG Operon, Ebersberg, Germany) were used to screen the variation among differently aged groups of *Silene* seeds. The RAPD–polymerase chain reaction (PCR) mixture consisted of 50 ng of template DNA, $1\times$ PCR buffer [7·5 mM Tris–HCl pH 9·0, 5·0 mM KCl, 2 mM $(NH_4)_2SO_4$], 400 μM dNTPs, 0·80 μM 10-mer random primer, 3 mM $MgCl_2$ and *Taq* polymerase (1 U; Biotools, Madrid, Spain) in a total volume of 25 μL. DNA amplification was carried out on a T Gradient apparatus (Biometra, Goettingen, Germany). Template DNA was initially denaturated at 94 °C for 3 min, followed by 40 cycles (1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C). Finally, extension was further prolonged for 10 min at 72 °C. For each reaction, three replicated samples were used in two independent experiments. Amplification products were separated by electrophoresis on 1·5 % agarose gels that were stained with ethidium bromide (0·5 μg L^{-1}; Sigma-Aldrich, Milan, Italy) and visualized under UV light. The 100 bp DNA Ladder and 1 kb DNA Ladder (GeneRuler, Fermentas, Burlington, Canada) were used as molecular standards.

The reproducible RAPD bands were scored in binary characters and coded accordingly (presence = 1, absence = 0). The binary data obtained were used to estimate Nei's unbiased genetic identity and genetic distance (Nei, 1972). This matrix was used to cluster samples into a dendrogram built with unweighted pair group method analysis (UPGMA). The analysis was performed using the POPGENE32 software.

Seed extract, DPPH test and determination of phenolic content

Seed extracts were prepared as described by Li *et al.* (2008). Seeds (60 mg) were reduced to fine powder using a pestle and a mortar; the sample was recovered by adding 1·2 mL of acetone: water (70:30; v/v) and then transferred to a microtube and incubated overnight at 23 °C under gentle shaking. Subsequently, the sample was centrifuged and the supernatant was recovered and stored at –20 °C.

DPPH (1,1-diphenyl-2-picrylhydrazyl) and the reference antioxidant (standard) ascorbic acid were obtained from Sigma-Aldrich (Milan, Italy). The free radical-scavenging activity of seed extracts was determined as described by Braca *et al.* (2001). The seed extract (100 μL) was added to 3 mL of a solution containing 100 μM DPPH dissolved in methanol. The reaction was carried out in the dark for 20 min at room

temperature. For each sample, absorbance at $\lambda = 517$ nm was measured using a V-530 spectrophotometer (Jasco Europe S.r.l., Cremella, Italy). Reduction of DPPH was calculated from $[(A_0 - A_1)/A_0] \times 100$ where A_0 is the absorbance of the control without seed extract and A_1 is the absorbance of the extract/standard. A standard curve was built using ascorbic acid with concentrations in the $0{\cdot}1$–$1{\cdot}5$ mM range, and the total antiradical capacity (TAC) was expressed as ascorbate equivalents (AEs) mg^{-1} d. wt.

The amount of total phenolic compounds was determined using the Folin–Ciocalteu reagent (Spanos and Wrolstad, 1990) and reported as gallic acid equivalents (GAEs) mg^{-1} d. wt by reference to a standard curve. The seed extract (20 μL) was mixed with deionized H$_2$O (1·58 mL) and with the Folin–Ciocalteu reagent (100 μL; Sigma-Aldrich). The sample was incubated for 1–8 min and the reaction was then neutralized with 300 μL of a sodium carbonate solution (w/v) (Na$_2$CO$_3$, 20 %; Sigma-Aldrich). The sample was incubated for 30 min at 40 °C in the dark. The absorption of the resulting blue colour was measured at $\lambda = 765$ nm, using a V-530 spectrophotometer (Jasco Europe S.r.l.). A calibration curve was built, using gallic acid with concentrations in the 50–400 mg L^{-1} range. The specific antioxidant capacity, defined as the ratio between the total antiradical capacity and the total phenolic content, was expressed as μg AE mg^{-1} GAE.

Quantitative real-time PCR (QRT-PCR) for gene expression analysis

RNA extraction was carried out using the Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad, Milan, Italy) and cDNA synthesis was then performed with the iScript cDNA Synthesis kit (Bio-Rad). QRT-PCR was carried out using the SsoFast^{TM} EvaGreen$^{®}$ Supermix (Bio-Rad) and a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia). The *Silene* housekeeping gene *EF1α* (elongation factor 1α) (GenBank accession no. GH294012) was used as standard control in the QRT-PCRs since it was reported to have the most stable expression among a wide range of conditions represented by different tissues and treatments (Nicot, 2005; Han *et al.*, 2012). To analyse the expression profiles of the *Silene SOD* (GenBank accession no. GH293101) and *MT2* (type 2 MT) (GenBank accession no. AF101825) genes, the oligonucleotide primers were designed using the Real Time PCR Primer Design program from GenScript (Table 1). QRT-PCR conditions were as follows: denaturation at 94 °C for 2 min, cycling at 94 °C (15 s), 58 °C (15 s), 72 °C (30 s). For each primer set, a no-template control was used. The QRT-PCR outputs of three biological replicates per sample and gene were analysed using the

LinRegPCR and REST 2009 computer software (Pfaffl *et al.,* 2002; Ramakers *et al.,* 2003). For each set of PCRs, the logarithms of the initial fluorescence (No) was calculated based on the individual PCR efficiency. For each reaction, the No values were normalized to the mean value of untreated replicated samples and then used for graphic representation.

Quantitative real-time PCR for telomere length analysis

Telomere length was evaluated with a QRT-PCR assay as described by Cawthon (2002) with the following modifications. In order to calculate the telomere absolute length, a standard curve was established using a 98-mer oligonucleotide containing 14 repeats of the plant telomere sequence TTTAGGG which was serially diluted from 10 ng to 10 pg. Telomere absolute length was expressed as log[TL(kb)], logarithm of telomere length (kb). Each reaction was performed in triplicate in a total volume of 25 μL containing 50 ng of DNA, 1× Maxima SybrGreen Master Mix (Fermentas, Burlington, Canada), 500 nM telomere forward primer (5′-CGGTTTGTT GTGGGTTGTGGGTTGTGGGTTGTGGGTTGTGGGTT-3′), 600 nM telomere reverse primer (5′- GGCTTGTCCTGACCC TTGACCCTTGACCCTTGACCCTTGACCCT-3′) and 0·5 % dimethylsulfoxide (DMSO; Sigma-Aldrich). The following cycle conditions were used: denaturation at 95 °C for 10 min; 15 s at 95 °C, 30 s at 56 °C and 1 min at 72 °C (40 cycles). Reactions were carried out in triplicate in a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia) and results were interpreted using the LinRegPCR computer software (Ramakers *et al.,* 2003).

Statistical analysis

Experiments were repeated three times and carried out in triplicate. Data are expressed as means ± standard deviation (s.d.) values. Differences observed in the *S. vulgaris* seeds as concerns the free radical-scavenging activity, total phenolic content, specific antioxidant activity, *SOD* and *MT* transcript accumulation, and telomere length, compared with the *S. acaulis* seeds were evaluated statistically. Statistical significance of differences was determined using Student's *t*-test ($*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$).

RESULTS

Effects of artificial ageing on the germination efficiency of S. vulgaris *and* S. acaulis *seeds*

When *S. vulgaris* and *S. acaulis* seeds were subjected to artificial ageing, significant differences were observed in terms

TABLE 1. *Sequences of oligonucleotide primers utilized in QRT-PCR*

Gene	Forward primer	Reverse primer	Efficiency*
SOD	5'-GAAGGAGATGGTCCAACAACT-3'	5'-TGTTTCCAAGGTCACCAGCA-3'	1·79
MT	5'-TGTGGATCTGCCTGCAAGT-3'	5'-ACACCCATTTCCATCTCTGC-3'	1·70
EF1	5'-TAACGGTTATGCCCCAGTTC-3'	5'-AGAAGGTCTCGACAACCATG-3'	1·66

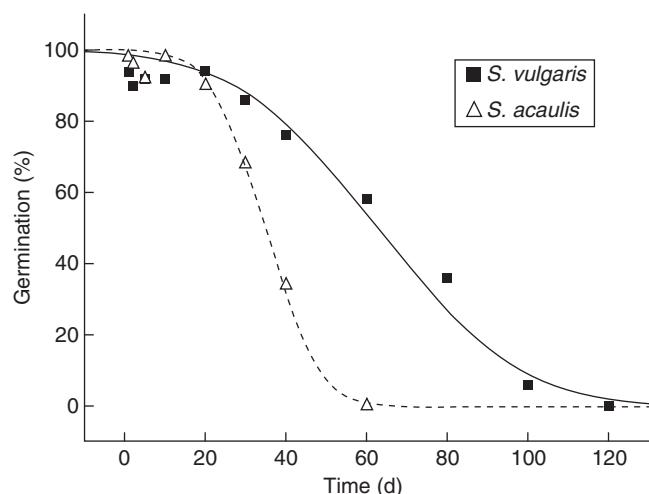*Efficiency of the primer pair in QRT-PCR

FIG. 1. Survival curves fitted by probit analysis for *Silene acaulis* and *S. vulgaris* seed lots. Seeds were subjected to experimental storage conditions at 45 °C, 60 % RH. All non-germinated seeds were cut-test at the end of the test and found to be dead.

of germination. In both the tested seed lots, germination declined as the period of experimental storage increased (Fig. 1). The two species differed in the time period required for seed germination to drop to 50 % of the T_0 value ($p50$) (approx. 35 compared with 63 d, Fig. 1). Variation in $p50$ values between the seed lots of *S. vulgaris* and *S. acaulis* was explained by differences in the initial seed viability (K_i; $F_{1,9} = 15.71$; $P < 0.01$) and by differences in the rate at which seeds lost the ability to germinate ($1/\sigma$, $F_{1,8} = 34.89$; $P < 0.01$). The reported data show the reduced seed germinability of the *S. acaulis* species.

ROS profiles in Silene *dry seeds collected from low and high altitude locations*

Electron paramagnetic resonance provides a unique opportunity for studying the reactive radical species, since it allows information to be acquired not only about ROS levels but also about the structure of the molecular environment surrounding the radical core. The EPR spectra of *S. acaulis* and *S. vulgaris* dry seeds are shown in Fig. 2A. Both spectra show a single peak characterized by a weak axial anisotropy with the peak to peak line width of 0.7 mT and $g_{//} = 2.0025$ $g_\perp = 2.0046$ for *S. acaulis* and the line width of 0.5 mT and $g_{//} = 2.0027$ $g_\perp = 2.0047$ for *S. vulgaris*. The comparison of normalized areas indicates that the concentration of radicals in *S. acaulis* is about 64 % of that found in *S. vulgaris*.

In order to better characterize the profiles of *S. acaulis* and *S. vulgaris* dry seeds, the samples were exposed to increasing microwave power and the resulting saturation behaviour was analysed (Fig. 2B). The areas of the EPR signals of *S. acaulis* and *S. vulgaris* dry seeds at the lowest microwave power were normalized for direct comparison of the power saturation curves. As shown in Fig. 2B, the signal recorded with the *S. acaulis* seeds increased progressively with the increasing microwave power, reaching an intensity of 5.0, 12.5 and 30.0 a.u. (arbitrary units) when 0.1, 1.0 and 10.0 mW power was provided. In contrast, the signal recorded with the *S. vulgaris*
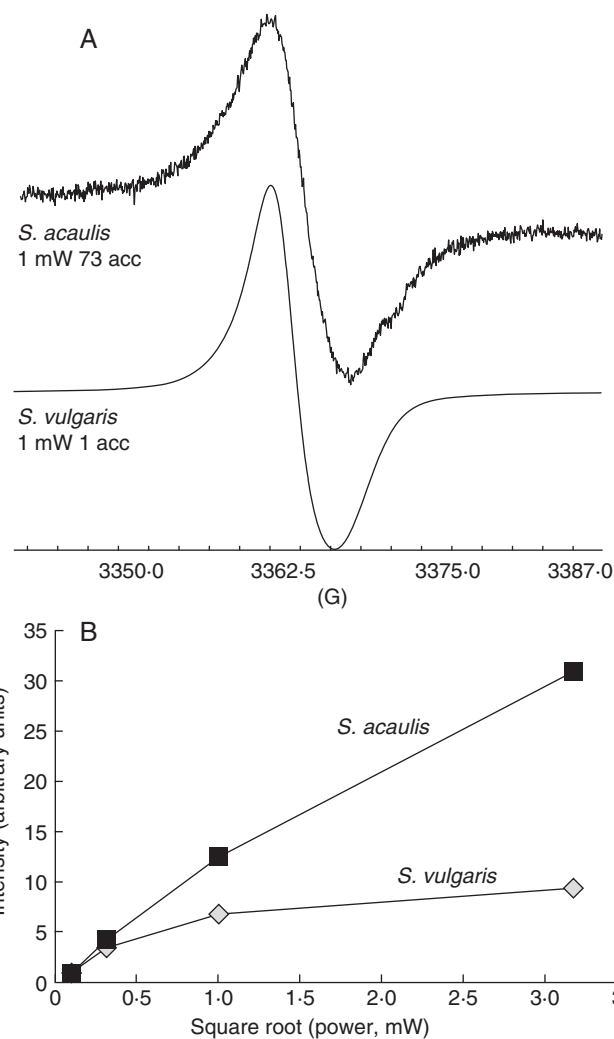

FIG. 2. (A) EPR spectra of *Silene acaulis* and *Silene vulgaris* dry seeds. The instruments settings were 1 mW for microwave power, a modulation amplitude of 0.2 mT, and ten and five accumulations for *S. acaulis* and *S. vulgaris*, respectively. (B) Power saturation curves of *S. acaulis* and *S. vulgaris* dry seeds. The areas of the spectra at 0.01 mW have been normalized to unity.

seeds increased up to 7.0 a.u. in response to 1.0 mW power and subsequently the signal intensity remained almost constant when a microwave power in the range 1–10 mW was applied. According to EPR analysis, the two *Silene* species showed a different saturation behaviour.

Effects of rehydration and artificial ageing on the RAPD profiles of S. vulgaris *and* S. acaulis *seeds*

Silene vulgaris and *S. acaulis* seeds collected from low- and high-altitude locations, respectively, were subjected to artificial ageing, and samples collected at the indicated time points (0, 5, 10, 20, 30 and 60 d) were used for molecular analyses. Dry seeds were also analysed, and the results were compared with those obtained from both the rehydrated and artificially aged seeds.

The RAPD profiles of *S. vulgaris* and *S. acaulis* seeds were analysed using five and four primers, respectively, out of 20 random primers screened individually in both species. In

TABLE 2. *RAPD primers producing bands with the* Silene *seed DNAs*

Species	Bands	OPA-9	OPA-16	OPA-17	OPA-18	OPA-19
S. vulgaris	Amplified	16	7	8	9	8
	Polymorphic	10	3	7	6	6
S. acaulis	Amplified	12	6	–	7	4
	Polymorphic	12	6	–	4	4

For each primer, the number of amplified bands and the number of bands resulting from polymorphic sites are indicated.
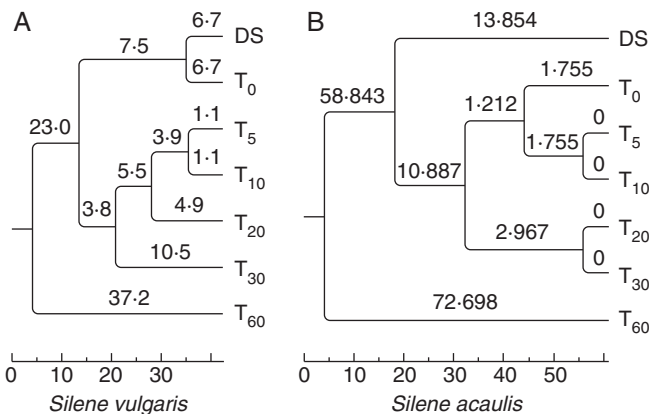

FIG. 3. UPGMA dendrograms corresponding to the RAPD profiles of dry seeds (DS) and artificially aged seeds belonging to (A) *Silene vulgaris* and (B) *S. acaulis*. Following rehydration, analyses were carried out at different time points (days) during artificial ageing (T_0, T_5, T_{10}, T_{20}, T_{30} and T_{60}).

S. vulgaris, five primers (OPA-9, OPA-16, OPA-17, OPA-18 and OPA-19) resulted in the amplification of bands when tested with DNA extracted from dry seeds and they produced polymorphic profiles. In the case of *S. acaulis*, four out of 20 primers (OPA-9, OPA-16, OPA-18 and OPA-19) revealed polymorphisms with dry and artificially aged seeds. Only four primers (OPA-9, OPA-16, OPA-18 and OPA-19) were able to amplify DNA bands in both species. The RAPD primers generated a total of 32 and 26 polymorphic fragments in *S. vulgaris* and *S. acaulis*, respectively (Table 2).

As for *S. vulgaris*, the UPGMA tree (Nei 1972) calculated from the RAPD data, divided the seed samples into three clusters (Fig. 3A). Both the dry seeds and the rehydrated seeds are grouped into one cluster, indicating that in *S. vulgaris* the RAPD profiles were not affected by rehydration, despite the fact that this step was carried out over 15 d. This finding might suggest that low levels of DNA damage and genomic instability are accumulated during rehydration in the *Silene* species originating from a low-altitude environment.

When considering artificially aged seeds, samples collected at 5, 10, 20 and 30 d are all grouped in the second cluster (Fig. 3A). This result is expected, since the level of DNA damage was enhanced as a consequence of artificial ageing. DNA alterations progressively accumulate during artificial ageing and, although the T_5 and T_{10} samples are grouped together within the second cluster, later on the genetic distance further increases since T_{20} and T_{30} samples are represented as single groups.

Also in the case of *S. acaulis*, the UPGMA tree calculated from RAPDs data separated the seed samples into three cluster (Fig. 3B). However, the dry seeds are grouped in a single cluster, while the rehydrated seeds and the artificially aged seeds are grouped in the second cluster. This finding suggests that the *S. acaulis* seeds undergo severe DNA rearrangements during the prolonged rehydration period. The results suggest that the *Silene* species originating from the high-altitude environment is less effective in controlling DNA damage, compared with *S. vulgaris*. When considering the artificially aged seeds, the T_5 and T_{10} samples are grouped together within the second cluster and subsequently the genetic distance further increases. In contrast to *S. vulgaris*, the T_{20} and T_{30} samples are grouped together. As previously reported for *S. vulgaris*, the extent of DNA damage was more pronounced at 60 d, as also indicated by the RAPD analysis, resulting in a visible smear after agarose gel electrophoresis (data not shown).

Cladograms of genetic distance obtained by RAPD analyses carried out in *S. vulgaris* and *S. acaulis* indicate distinct trends associated with rehydration and artificial ageing, showing the different capacities of the two species to overcome the problem of DNA damage. The reported data strengthen the use of RAPD as a reliable tool for investigating seed quality as affected by prolonged storage.

Correlation between the radical-scavenging ability and the content of total phenolics in Silene *seed extracts*

Besides the basal antioxidant features of the dehydrated seed, enhanced protection against radical species also results from the activation of ROS-scavenging mechanisms during imbibition. For this reason, the antioxidant profile of *Silene* seeds was analysed with biochemical assays in the dry and rehydrated seed samples. The radical-scavenging ability of *S. vulgaris* and *S. acaulis* seed extracts was measured using the DPPH test, expressed as μg AE mg^{-1} d. wt. For each species, extracts from dry seeds and rehydrated seeds were analysed and compared, as shown in Fig. 4A. In the case of *S. vulgaris*, the estimated DPPH-scavenging activity of dry seed and rehydrated seed extracts was 5.62 ± 0.12 and 6.43 ± 0.11 μg AE mg^{-1} d. wt, respectively. When compared with *S. vulgaris*, both the dry and rehydrated seed extracts of *S. acaulis*, revealed significantly ($P < 0.0025$) lower values of DPPH-scavenging activity, corresponding to 4.24 ± 0.01 and 4.33 ± 0.22 μg AE mg^{-1} d. wt, respectively (Fig. 4A). This might suggest that the *S. acaulis* seeds, collected at high altitude, possess a limited antioxidant response while,
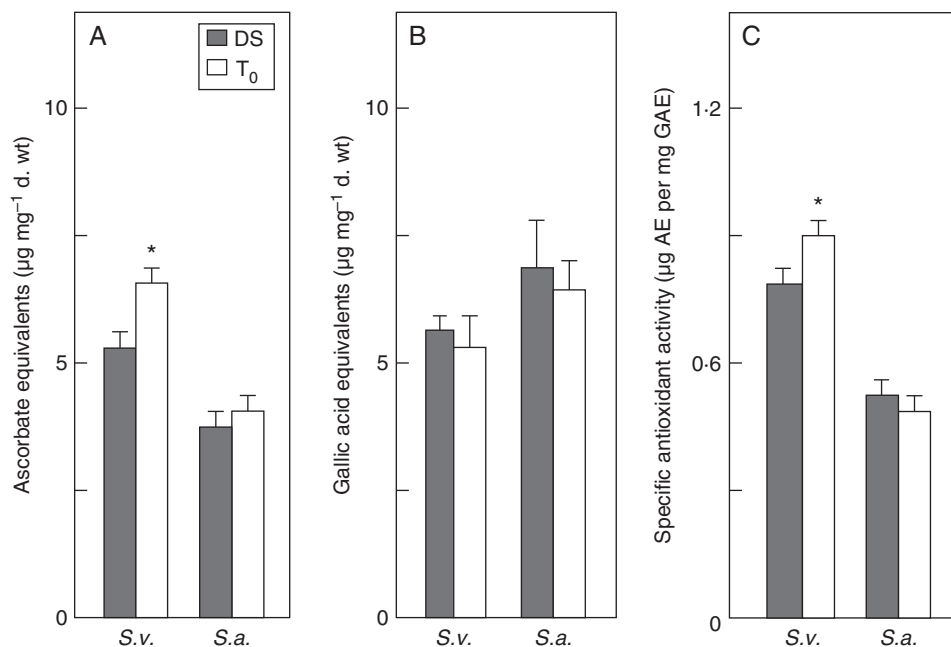
Fɪɢ. 4. Antioxidant properties of *Silene vulgaris* (*S.v.*) and *S. acaulis* (*S.a.*) seed extracts. (A) DPPH radical-scavenging activity expressed as µg of ascorbate equivalents per mg of dry weight. (B) The total phenolics content, evaluated using the Folin–Ciocalteu reagent method, expressed as gallic acid equivalents per mg of dry weight. (C) Specific antioxidant activity is expressed as µg ascorbate equivalents per mg gallic acid equivalents. Values are expressed as means ± s.d. of three independent experiments. Statistical significanc: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, compared with *S. acaulis*. DS, dry seeds. T_0, rehydrated seeds.

interestingly, the highest DPPH-scavenging ability was found in the extracts from rehydrated seeds of the *Silene* species living at low altitude. Furthermore, in the case of *S. acaulis*, no significant differences in the radical-scavenging activity of dry and rehydrated seeds were detected.

Since the reduction of DPPH can result from the radical-scavenging activity of phenolic compounds, such as flavonoids, polyphenols, tannins and phenolic terpenes, the total content of phenolic compounds was measured in the *Silene* seed extracts. Results from this analysis are shown in Fig. 4B. The estimated content of total phenolic compounds in *S. vulgaris* seed extracts in the dry and rehydrated state was 6.59 ± 0.00 and $6.46 \pm 0.36\ \mu g\ GAE\ mg^{-1}$ d. wt, respectively. Apparently, no significant differences were observed in the amount of total phenolics in both dry and rehydrated seed extracts of the species from the low-altitude location. As for *S. acaulis*, similar results were obtained, since the content of total phenolic compounds in the dry seed extract ($7.31 \pm 0.93\ \mu g\ GAE\ mg^{-1}$ d. wt) did not differ significantly from that measured in the rehydrated seed extract ($6.86 \pm 0.25\ \mu g\ GAE\ mg^{-1}$ d. wt) (Fig. 4B).

Finally, results from the DPPH test and from the Folin–Ciocalteu reagent method were combined to calculate the specific antioxidant activity of the *Silene* seeds, expressed as µg AE mg^{-1} GAE (Fig. 4C). Based on this calculation, the specific antioxidant activity of seed extracts was significantly enhanced in the *S. vulgaris* species found in a low-altitude location, compared with the species collected at high altitude. As shown in Fig. 4C, in *S. vulgaris* the specific antioxidant activity was $0.86 \pm 0.00\ \mu g\ AE\ mg^{-1}$ GAE in the dry seed extract and it further increased, up to $0.98 \pm 0.03\ \mu g\ AE\ mg^{-1}$ GAE, in rehydrated seeds. On the other hand, the dry seed extract of

S. acaulis revealed a specific antioxidant activity of $0.62 \pm 0.02\ \mu g\ AE\ mg^{-1}$ GAE and no significant differences were observed in rehydrated seeds which showed an estimated value of $0.58 \pm 0.03\ \mu g\ AE\ mg^{-1}$ GAE.

The reported data suggest that the two *Silene* species possess different antioxidant capacities, the high-altitude species being characterized by a lower radical-scavenging activity. Furthermore, it seems that the high content of total phenolic compounds observed in the dry seed extract of *S. acaulis* does not support the radical-scavenging ability.

Distinct expression profiles of the *SOD and* MT2 *genes in* S. vulgaris *and* S. acaulis *seeds*

The expression profiles of the *SOD* and *MT2* genes encoding the cytosolic SOD and a type 2 MT, respectively, were chosen as molecular indicators of the antioxidant response in *Silene* seeds. The expression patterns of the *SOD* and *MT2* genes were investigated only in dry seeds and rehydrated seeds since imbibition appears to be a critical step for the activation of antioxidant genes that provide protection during germination. Results from these experiments are shown in Fig. 5. As regards *S. vulgaris*, both genes were up-regulated during seed rehydration. The level of *SOD* transcript was significantly ($P < 0.05$) increased (up to 15-fold) in rehydrated seeds, compared with dry seeds, while the *MT2* gene expression was enhanced up to 100-fold (Fig. 5). In *S. acaulis*, no fluctuations in the amount of the *SOD* mRNA was observed in dry seeds, compared with rehydrated seeds, while the level of *MT* transcript significantly increased (up to 58-fold) in the rehydrated seeds of *S. acaulis* (Fig. 5).
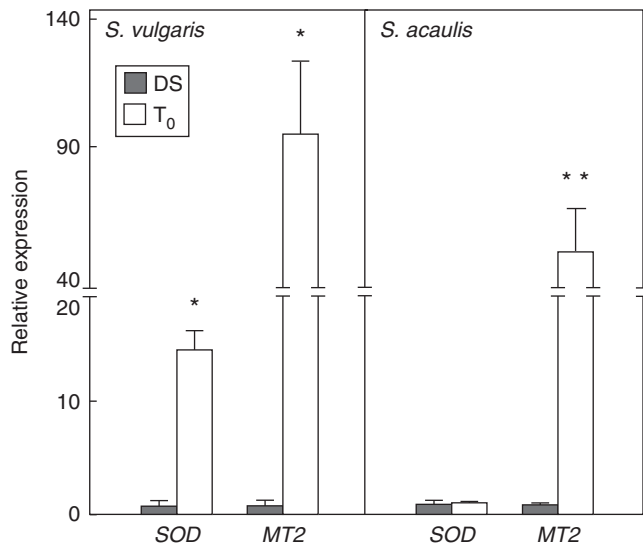
FIG. 5. Expression profiles of the *SOD* and *MT* genes in *Silene* seeds were evaluated by QRT-PCR. Values are expressed as means ± s.d. of three independent experiments. Statistical significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, compared with *S. acaulis*. DS, dry seeds; T_0, rehydrated seeds.
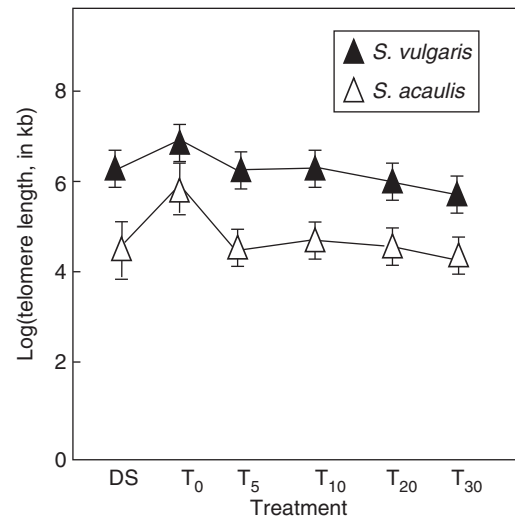

FIG. 6. Telomere length measurement by a QRT-PCR-based approach in *Silene vulgaris* and *S. acaulis* dry seeds (DS), rehydrated seeds (T_0) and artificially aged seeds collected at different time points (5, 10, 20 and 30 d; T_5, T_{10}, T_{20} and T_{30}). Data were log transformed, and values are expressed as means ± s.d. of three independent experiments.

The QRT-PCR analysis provided useful information concerning the ability of *Silene* seeds to activate the antioxidant response at the transcriptional level, and both *SOD* and *MT2* genes might be used to screen other members of the *Silene* genus.

Telomere length homeostasis in S. vulgaris *and* S. acaulis *seeds*

The usefulness of telomere length as a parameter for assessing seed deterioration is still debated, while the current knowledge needs to be expanded. For this reason. telomere length was measured not only in dry and rehydrated seeds of *S. vulgaris* and *S. acaulis* but also during artificial ageing, using QRT-PCR. Results from these analyses are shown in Fig. 6.

The estimated telomere length in dry seeds was 6.09 ± 0.05 and 5.31 ± 0.19 log[TL(kb)] per reaction in *S. vulgaris* and *S. acaulis*, respectively. This finding highlights a significant difference between the two species in telomere length. In both *S. vulgaris* and *S. acaulis*, telomere length significantly increased following rehydration. As shown in Fig. 6, the estimated telomere length in *S. vulgaris* rehydrated seeds was 6.30 ± 0.04 log[TL(kb)], 2-fold higher compared with dry seeds. In the case of *S. acaulis*, the observed enhancement in telomere length was 5.99 ± 0.13 log[TL(kb)] in rehydrated seeds, 3-fold higher compared with dry seeds.

During artificial ageing, a significant reduction in telomere length was observed in both species compared with rehydrated seeds (Fig. 6). After 5 d of artificial aging (T_5), *S. vulgaris* showed an average telomere length of 6.08 ± 0.05 log[TL(kb)], and subsequently values of 6.19 ± 0.05, 6.14 ± 0.01 and 5.98 ± 0.01 log[TL(kb)] were measured at 10, 20 and 30 d of treatment (T_{10}, T_{20} and T_{30}). In the case of *S. acaulis*, the average telomere length was 5.33 ± 0.05 log[TL(kb)] at T_5. Subsequently, values of 5.46 ± 0.03, 5.40 ± 0.01 and $5.34 \pm$

0.04 log[TL(kb)] were measured at 10, 20 and 30 d of treatment (T_{10}, T_{20} and T_{30}).

The results presented here highlight significant species-specific differences in telomere lengthening between *S. vulgaris* and *S. acaulis*. Seed rehydration resulted in telomere lengthening in both *Silene* species. The dynamics of telomere shortening in relation to seed ageing represent an interesting and novel aspect of seed physiology that deserves more in-depth investigation.

DISCUSSION

The present work addresses some molecular aspects related to the *ex situ* preservation of short-lived seeds, particularly those originating from high-altitude environments. For seeds stored under identical conditions (60 % RH, 45 °C), there was a significant variation in seed longevity between *S. vulgaris* and *S. acaulis* inhabiting low- and high-altitude locations, respectively. The estimated $p50$ values ranged between 35 d (*S. acaulis*) and 63 d (*S. vulgaris*), confirming the findings of Mondoni *et al.* (2011, 2012) that alpine species are significantly shorter lived than lowland counterparts. Although seeds of both species were collected at the moment of natural seed dispersal and then stored under identical conditions, caution is needed when interpreting differences in seed longevity solely due to the role of species and habitat, as initial seed quality may also play a role.

The *Silene* seeds showed EPR spectra composed of only one weak singlet line which is due to the presence of radicals with a quinoide nature (Yordanov *et al.*, 2004). The Q-band EPR study carried out by Yordanov *et al.* (2004) demonstrated the anisotropic nature of this species having *g*-values compatible with radicals from polyphenols, normally found as antioxidant compounds within plant tissues (Swartz *et al.*, 1972; Nandi *et al.*, 1997). When high and low vigour rice (*Oryza sativa* L.) dry seeds stored in natural environment were analysed by

EPR spectroscopy, loss in viability due to high temperature and humidity correlated with a decrease in the level of free radicals, possibly proposed to be carbon-based quinone derivatives (Nandi *et al.*, 1997). It was hypothesized that cell deterioration in the rice embryonic axis depends on the balance between free radical accumulation and the activity of ROS scavengers, e.g. the antioxidant enzyme SOD. The saturation curves, obtained by challenging the *Silene* dry seeds with increasing microwave power, provided valuable information on both the radical structure and their surrounding environments. In fact if the radical species are free to move and interact with the other molecules, they are able to absorb more microwave power, thus reaching saturation at higher energy values. In contrast, if the radical species are in a stabilized form, the saturation level is rapidly reached. In the case of *S. acaulis*, no saturation was observed in the tested power range and this might be due to the highly efficient energy transfer occurring from the excited state to the environment surrounding the radical species. Moreover, the efficiency of energy transfer increases with the number of molecular interactions between the radical species and the surrounding environment. When considering the phenolic compounds and their radicals, the most relevant interaction is mediated by hydrogen bonds and it is directly related to the polarity of the medium, thus to the water content as well as to the presence of polar structures. Increased water content inevitably leads to enhanced radical mobility and decay, and this might explain the finding highlighted by EPR that the *S. acaulis* dry seeds show a lower radical content, compared with *S. vulgaris*. On the other hand, the *S. vulgaris* seeds exposed to increasing microwave power rapidly reached saturation in terms of signal intensity. Such a response might be due to the presence of a non-polar environment surrounding the radical species and, possibly to a reduced water content. The reported data are in agreement with the biochemical profiles provided by the analysis of the radical-scavenging ability and the content of total phenolics in the *Silene* seeds. They also highlight the potential of EPR technology as an effective and low-cost tool for investigating the radical profiles in seeds and the surroundings of the radicals. A more detailed EPR analysis carried out with individual seeds might help in clarifying the spatial distribution of free radicals as demonstrated for several legume and *Brassica* species (Hepburn *et al.*, 1986).

In accordance with the observed variation of seed longevity, the additional analyses reported here demonstrate the occurrence of variation in the RAPD profiles of genomic DNA extracted from dry, rehydrated and artificially aged seeds of both species. Thus, the molecular approaches used in the present work allowed discrimination between the seed response to rehydration and artificial ageing.

At the moment, we can only speculate about the extent of DNA repair in the *Silene* seeds and we do not know how ageing could affect these crucial functions, paving the way to increased global damage. As already highlighted by Vijay *et al.* (2009), different responses have been reported concerning the qualitative and quantitative distribution of DNA lesions during seed ageing. This is the case of artificially aged rye embryos in which Boubriak *et al.* (1997) showed an accumulation of DNA nucleosome multimers rather than random fragmentation. Besides this, the occurrence of chromatin remodelling events, e.g. histone modifications (Tanaka *et al.*, 2008; Filkenstein *et al.*, 2008), that might be responsible for the observed RAPD profiles, cannot be ruled out.

In the case of *S. vulgaris*, both dry and rehydrated seeds are grouped in the same cluster, leaving the artificially aged seeds in a separate cluster. This means that the *Silene* species originating from a low-altitude environment produces seeds with the ability to withstand the oxidative injury that inevitably takes place during imbibition. This is not the case for *S. acaulis*, the species from high elevations, since consistent changes in DNA profiles were detected when dry seeds were compared with rehydrated seeds. The relationship between DNA damage and the seed moisture content has been recently established by El-Maarouf *et al.* (2011) who used RAPD analysis to demonstrate the threshold moisture value at which DNA laddering, a typical hallmark of programmed cell death, occurred. In the artificial ageing experiments, the aim of the rehydration step is to raise the moisture content of the seeds prior to ageing and to minimize the subsequent adjustment of moisture content when samples are transferred to the ageing conditions (Davies and Probert, 2004). In our study, in place of seed moisture content measurement, we have considered the eRH (equilibrium relative humidity) as an alternative, non-destructive method to monitor the level of seed imbibition, when seeds were moved from the drying room (15 % RH) to the rehydration environment (47 % RH).

The present work demonstrates that seeds of *S. acaulis* from alpine locations are particularly vulnerable to oxidative damage when imbibition is carried out, indicating that seed viability was already affected in this species, before transfer to the artificial ageing environment. Based on this evidence, the timing and/or conditions of the rehydration phase should be reconsidered when comparing short-lived seed lots. From this point of view, the RAPD markers tested here might be used to work out improved rehydration protocols.

It has also been demonstrated that germination efficiency positively correlates with the level of free radical scavenging or antioxidant potential, used as an indicator of seed longevity (Bailly *et al.*, 1998). More recently, Talai and Sen-Mandi (2010) have evaluated the total antioxidant potential of rice (*O. sativa* L.) seeds and found that freshly harvested seeds with fast germination possess a higher total antioxidant potential, compared with aged seeds. Birtic *et al.* (2011) demonstrated that the half-cell reduction potentials of low molecular weight thiols, cysteine, cysteinyl-glycine and γ-glutamyl-cysteine, can be used as markers of seed ageing.

According to the reported data, the highest antioxidant activity was recorded in extracts of rehydrated seeds of the low-altitude species *S. vulgaris*. Furthermore, the high antioxidant activity of the *S. vulgaris* seed extracts correlated with the amounts of total phenolic compounds, suggesting that, in this species, phenols might act as antioxidant molecules. In contrast, the analyses carried out on the *S. acaulis* seed extracts revealed that high levels of total phenolic compounds, found in dry seeds, were not associated with increased antioxidant properties. This might be related to the chemical nature of the phenolic molecules accumulated in the *S. acaulis* seeds. This aspect of seed physiology has been recently highlighted by Mhamdi *et al.* (2010), who found that the antioxidant activity of seed extracts of *Borago officinalis* depends on the

composition rather than content of total phenolics. Accumulation of phenolics is more generally considered as a protective mechanism of plants against adverse environments, and increased levels of phenolics have been measured during the last stages of seed maturation in relation to increasing temperature (Mhamdi *et al.*, 2010). According to the reported data, the specific antioxidant activity of seed extracts might be a promising viability marker.

The seed antioxidant response can also be evaluated at the molecular level, by monitoring the expression profiles of antioxidant genes. The SOD enzyme, involved in the early scavenging of superoxide radicals, is an essential component of the seed antioxidant system and, accordingly, the *SOD* gene expression is responsive to seed imbibition under physiological conditions and osmotic stress (Balestrazzi *et al.*, 2010; Macovei *et al.*, 2011*b*). Moreover Mylona *et al.* (2007) demonstrated that exposure of maize scutella to ROS-generating xenobiotics results in the accumulation of *SOD* transcript, which correlates with the increase in the enzyme activity. Recently, Yao *et al.* (2012) reported the presence of *SOD* transcript in pea dry seed and accumulation during imbibition. The same authors also observed a decline in *SOD* expression as a consequence of seed ageing which correlated with reduction in seed germinabilty and viability. Thus they suggest a key role for SOD in mediating the deleterious effects of ageing. In accordance with these observations, the level of *SOD* transcript is indicative of the antioxidant response in *Silene* seeds, since significant accumulation was detected only in the low-altitude species. The *MT2* gene, encoding a type 2 MT, typically induced by oxidative stress, turned out to be upregulated, even more than the *SOD* gene, in both species. Although there are no reports on the expression profiles of *MT* genes in *Silene* seeds, it is known that the genus *Silene* includes several heavy metal-tolerant species characterized by high levels of *MT* transcript (van Hoof *et al.* 2001; Mengoni *et al.*, 2001). In a recent work, Zhou *et al.* (2012) demonstrated that transgenic arabidopsis seeds overexpressing the *NnMT2a* and *NnMT3* genes from sacred lotus (*Nelumbo nucifera* Gaertn.) show improved resistance to accelerated ageing, thus indicating the protective role of MTs.

The two *Silene* species showed significant differences in their average telomere length. This finding is in agreement with the current literature which highlights the involvement of species-specific factors in the regulation of telomere length. Ecotype-specific telomere lengths have been reported in arabidopsis as a result of genetic and/or epigenetic differences affecting the balance between telomere shortening/extension (Maillet *et al.*, 2006).

A few studies carried out in wheat and rye seeds have shown the correlation between telomere length and seed ageing, demonstrating the fragmentation of chromosomal telomeric sequences during ageing (Bucholc and Buchowicz, 1995; Boubriak *et al.*, 2007). Moreover, it has been reported that restoration of telomeres at chromosome ends takes place early during seed imbibition, possibly due to the presence of telomerase activity in dry seeds (Riha *et al.*, 1998).

An interesting aspect of the *Silene* seed response was the increased telomere length observed in the rehydrated seeds, particularly evident in *S. acaulis*, the species from a high altitude with lower antioxidant ability. This process might be related to seed repair mechanisms, which are activated during the early phase of seed imbibition to remove DNA fragmentation (Balestrazzi *et al.*, 2010, 2011*a*). This hypothesis has been strongly supported by Bucholc and Buchowicz (1992) who demonstrated that dry wheat embryos subjected to long-term storage were depleted of telomeric repeats, while an enrichment was evident at the beginning of imbibition. On the other hand, there are reports describing the effects of altered telomere homeostasis on seed germinability that suggest the association between increased telomere length and genotoxic stress. The *Arabidopsis rtbp1* (*RICE TELOMERE BINDING PROTEIN1*) mutant, characterized by increased telomere length compared with the wild type, was severely impaired in seed germination (Hong *et al.*, 2007). Similarly, the lack of the k70 function required for telomere maintenance resulted in the marked expansion of telomere length and a high sentitivity to genotoxic agents (Riha *et al.*, 2002). This is a very intriguing perspective which deserves additional investigations. A model has recently been proposed in animal cells, where telomere oxidative damage resulting from endogenous or exogenous sources can be differently recognized and induce different responses (Wang *et al.*, 2010). According to these authors, moderate injuries in telomeric sequences caused by cellular metabolism are known to activate telomere lenghtening, while extensive damage caused by external factors result in telomere degradation. In agreement with the model, when considering seeds, telomere lenghtening might be expected after rehydration when there is resumption of metabolic activities, whereas telomere degradation might be associated with artificial ageing.

In the present work, we have selected different markers of seed deterioration that might be useful to predict seed viability not only in plant species from different altitudes but, possibly in a wide range of endangered species undergoing *ex situ* preservation. As evidenced, each molecular marker is related to specific cellular responses, including DNA damage/repair and the antioxidant system. RAPD analysis, quantitation of redox activity of non-enzymatic antioxidant compounds and gene expression profiling can be used to acquire more in-derpth information on the eco-physiological features of *Silene* species, as low-cost and time-saving reproducible procedures. Moreover, considering the limited knowledge currently available, telomere analysis proved to be a promising tool that can be applied to a large number of seed samples. A positive impact of the reported results could be envisaged within a relatively short time, since specific suggestions can be derived for improving the rehydration protocol of seeds from a high-altitude location.

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