

Full Length Research Paper

Effects of natural long storage duration on seed germination characteristics of *Periploca angustifolia* Labill.

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This study was carried out to evaluate the effect of long-term natural aging on germination ability and several biochemical characteristics regarding soluble sugars and polyphenol matter contents and radical scavenging activity of *Periploca angustifolia* Labill. (Asclepiadaceae) stored seeds for 1, 3, 7, 10, 11 and 15-years, dry storage compared to freshly collected seeds. The long-term aging caused an important decrease of germination percentage, seed vigor index, seeds' viability, moisture content and seed vigor except for seeds stored for seven-years. The latter showed the highest percentages of germination and viability, seed vigor index and seed vigor under a 7.3% moisture content when compared to the oldest seeds (15-years old) which presents the lowest moisture content. In our study, aged seeds showed the lowest radical scavenging percentage activity and amounts of polyphenol, keeping free radicals and peroxides at high levels causing thereby seeds deterioration. *P. angustifolia* seeds undergo a process of after-ripening under the storage conditions, possibly depending on the low, but steady water loss down to an optimal storage water content of approx. 7.3%, thereafter undergoing some deterioration as indicated by reduced amounts of soluble sugars by polyphenol contents and experimentally tested antioxidant activities, which is in line with increased membrane leakages as indicated by increased electrical conductivities of solution from experimentally soaked seeds.

Key words: *Periploca angustifolia* Labill, polyphenol, germination.

INTRODUCTION

The necessity of seed storage was firstly recognised when humans began to domesticate plants thousands of years ago. Viable seeds had, and still have, to be maintained from one growing season to the next and the necessity of seed conservation (*in-situ* or *ex-situ*) is one of the best strategies for the protection of plant diversity (Rajjou and Debeaujon, 2008). The goal of gene banks is to maintain seed viability for indefinite periods of long

term storage, typically for 10 to 100-years or more. According to Rajjou and Debeaujon (2008), seed longevity varies among, and within species due to the differences in genotype, provenance and the cumulative effects of the environment.

Understanding the germination process will help in the conservation strategies. Seed germination considered as a critical phase in the reproductive cycle of great importance for species fitness, and variation in germination percentage has been interpreted as an adaptation to ecological conditions (Navarro and Guitián, 2003; Rajjou and Debeaujon, 2008). In the context of climate change, plant genetic composition may change in response to the selection pressure and some plant communities or

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species associations may be lost as species move and adapt at different rates (Donohue et al., 2005). Seeds of many plant species are extremely tolerant to harsh environmental conditions provided they are in a state of desiccation. In this dry state, their metabolic activity is drastically reduced to a very low level (quiescence) while retaining their ability to germinate for considerable periods (Buitink and Leprince, 2008).

Active oxygen species (AOS) are involved in various aspects of seed physiology (Quan et al., 2008). Their generation, which occurs during seed desiccation, germination and ageing, may lead to oxidative stress and cellular damage, resulting in seed deterioration (Møller et al., 2007). However, cells are endowed with detoxifying enzymes and antioxidant compounds that scavenge AOS and participate in seed survival (Bailly et al., 2008). The detoxifying mechanisms play a key role in acquisition of desiccation tolerance of developing seeds, completion of seed germination and seed storability (Garnczarska et al., 2009). Accumulation of antioxidant components in dry seeds during the tardy maturation step on the mother plant contributes to control their storability potential (Bailly, 2004).

The protective role of antioxidant secondary metabolites such as total phenols and soluble sugars during aging or oxidative stress is well documented. Knowing and understanding the complex features that govern seed longevity are therefore of major ecological, agronomical and economical importance.

Periploca angustifolia Labill. (Asclepiadaceae) is a perennial shrub widely distributed in southern Spain, Silice and North Africa. It is a Saharan-Mediterranean species common in semi-arid and arid bioclimatic zones of Tunisia (Le Floc'h et al., 1989). This species is an evergreen shrub with a branching bush-type that reaches 3.5 to 4 m and dominant in active sand dunes and stabilized sand fields in southern Tunisia (Neffati, 1994). It is browsed by livestock and its leaves are valuable animal forage and fodder under open grazing conditions (Neffati, 1994). Le Floc'h (1983) reported that a decoction of leaves is as sweet as tea and that the roots are used as a source of steroids. The roots are used for medicinal purposes as treatment against hypertension Ferchichi (1990) and as antidiabetic (Askri, 1988). These characteristics gave to *P. angustifolia* a valuable multi-purpose shrub for semi-arid to arid ecological areas.

The present study was undertaken to provide basic information on life span of *P. angustifolia* seeds, to verify the possibility of their conservation and to recognize possible relationships between seed viability, germination, enzymatic and non-enzymatic antioxidants. The polyphenol and soluble sugar contents and radical scavenging activity were determined over the storage period of the seeds. Also, the focus of the work is to study the after-ripening phenomenon under long-term seed storage. Information from this study will be used in establishing

programs for reintroduction of this shrub in arid and semi-arid regions of Tunisia as a measure to rehabilitate degraded lands.

MATERIALS AND METHODS

Seed collection

Seeds were collected from 12 plants in early June at the experimental site of the Arid Land Institute-Medenine (El Fjé 65°25'E, 37°07'N; South-East Tunisia). This area is arid to semi-arid with a typical Mediterranean climate, characterized by irregular rainfall events and a harsh dry summer and cold winter periods. Annual rainfall is around 144 mm and annual mean evapotranspiration 1096 mm. Mean annual temperature is 20.5°C with a minimum temperature 6.2°C in January and 36.8°C maximum in August (Gorai and Neffati, 2007). Seeds were stored in air-dry storage in the seed bank of the Range Ecology Laboratory in which relative humidity was set at 30% and temperature was maintained at 20°C. The seeds used dated from 1994 to 2009. Seeds collected in June, 2009 were considered as controls compared to other dates of collect (1994, 1998, 1999, 2002, 2006 and 2008 that correspond to 15, 11, 10, 7, 3 and 1-year aged seeds, respectively).

Germination experiments

Seeds were surface sterilized with sodium hypochlorite prior to any experimental usage to avoid fungal attack. Petri dishes (90 mm) containing two disks of Whatman filter papers soaked in distilled water. During ten days, germination experiments were conducted in an incubator at 25°C in complete darkness (Luminincube II, analis, MCAS-350, Belgium). A completely randomized design in the germination tests (five replicates of 20 seeds) was used. Petri dishes were inspected daily until the first seed had germinated to identify the onset of germination and distilled water was added, if necessary, to keep the paper moist. Seeds were scored as germinated when at least 2 mm of the radicle was visible.

Seed viability test

Seed viability was evaluated by measuring the respiratory capability of isolated embryos according to Tommasi et al. (2006) slightly modified method. The isolated embryos (25) were incubated for 1 h in 5 mL of a solution of 1% (w/v) 2,3,5 triphenyl tetrazolium chloride (TTC) in phosphate buffer 50 mM, pH 7.3 for 3 to 4 h at 25°C in darkness. Seeds of which the embryo exhibited no overall carmine red staining were scored as non-viable. Viability percentage (VP) was calculated as the number of viable embryos/total number of embryos × 100.

Determination of seed vigor (SV)

The electrical conductivity (EC) of the soak water of stored seeds was measured according to Goel et al. (2003) with slight modification. Three replicates, each one was carried out with 50 seeds which were weighted and then soaked in 5 mL of deionised water at 20°C for 24 h and the EC of the seed soak water determined using a conductivity meter (INOLAB). The EC was expressed as $\mu\text{S m}^{-1} \text{seed}^{-1}$.

Moisture content (MC)

Moisture content (MC) was estimated by drying 100 mg of seeds for

24 h at 105°C. The MC (mL g^{-1} FW) = (FW-DW/FW) × 100 was determined on a fresh weight basis; where, FW is the fresh weight and DW is the dry weight. All measurements were done in triplicates.

Methods of germination expression

From the germination data collected, the following variables were determined according to these formulas:

1. Germination (%) = (number of germinating seeds/total number of seeds) × 100.
2. Germination index = $\Sigma G/t$; where, G is the relative germination percentage at two-day intervals and t is the total germination period.
3. Vigor index (VI) = $G \times S_x$; where, G is the germination index and S_x is the radical mean length 10 days after germination (Yi et al., 2008).

Total phenol assay

The total phenols in seeds were determined by the Folin-Ciocalteu method as described by Li et al. (2007) with some modifications. A lot of 200 mg of seeds were homogenized with 5 mL of methanol. A volume of 1 mL of Folin-Ciocalteu's (10 X) reagent was then added to the 200 μL of the extract and stirred vigorously by vortex and left to stand for 5 min. Finally, 800 μL of saturated sodium carbonate solution (5%) was added, stirred vigorously and left to stand in darkness at room temperature for 2 h. Absorbance was measured at 765 nm using a spectrophotometer (Jenway). Determination of total phenols was carried out in triplicates, and the results are mean values, calculated on the starting material weight basis and given as μg of gallic acid equivalent (GAE) per gram of seed FW from the calibration curve of acid gallic standard solutions (0 to 25 $\mu\text{g mL}^{-1}$).

2,2-Diphenyl-1-pycrilhydrazil hydrate (DPPH) radical scavenging activity assay

DPPH (2,2-diphenyl-1-pycrilhydrazil hydrate) radical scavenging activity was determined according to the method of Amarowicz et al. (2004) that is slightly modified by Yesil-Celiktas et al. (2007). A lot of 100 mg of seed FW was homogenized with 2 mL of methanol and centrifuged at 9,000 g for 15 min. To 100 μL of extract, 2.9 mL of 0.1 mM methanolic solution of DPPH was added. The contents were stirred vigorously and then left to stand at room temperature for 30 min in dark. Decrease in colorization was measured spectrophotometrically at 517 nm. The radical scavenging activity (RSA) was calculated using the equation: RSA (%) = $100 \times (1 - AE/AD)$; where, AE represent the absorbance of the solution containing antioxidant extract and AD is the absorbance of the DPPH solution. All measurements were done in triplicates.

Determination of soluble sugars

Soluble sugar was determined following the phenolsulphuric acid method according to (Robyt and White, 1987). 100 mg of seeds were grinded in fine powder and moistened in 5 mL of methanol (80%) then incubated at 70°C for 30 min. The extract was centrifuged at 5,000 rpm for 10 min. To 1 mL of supernatant, 1 mL of 5% phenol and 5 mL of concentrated sulphuric acid, were added.

Optic density was read by a spectrophotometer at 490 nm. Soluble sugar concentrations were expressed as mmol g^{-1} seed FW using D-glucose as standard. All measurements were done in triplicates.

Statistical analysis

All data analyses were performed with the software SPSS 13.0 (SPSS Inc, Chicago, Illinois, USA). Analysis of variance was used to assess the effect of storage period on the studied parameters. Statistical comparisons between means were performed with Student-Newman-Keuls' test. Differences were considered significant at $P < 0.05$. The Pearson Correlation was used to establish relationships between the measured parameters.

RESULTS

Germination percentages

As shown in Figure 1a, the seeds collected in June 2009, considered as controls in entire study, reached a germination percentage of 56%. When compared to control and other storage period, the highest germination percentage (93%) was attained for seven-year old seeds. After one to three-year storage period, germination percentages were lower (72 and 81%, respectively) than those after seven-years and did not show any significant differences, whereas they became significantly higher than control. The lowest germination percentage was obtained after eleven-year storage period (31%). However, no germination was recorded after 15-years of storage. Also, all seeds of the different storage periods showed the same delay of germination (two days). However, 11-years aged seeds germinated after one day. The curve of survival presented in Figure 1b that represent the mean final germination to the period of storage, showed that seeds longevity decreased after seven-years of storage. Before this period, final germination percentage increased significantly to reach 93%.

Seed viability

Due to the storage period, the overall seed viability decreased except those recorded after seven years (Table 1). Before the storage, seed viability was 80%, while it decreased significantly by increasing the storage time (78.67, 73.33, 45.33, 41.33 and 24% at 1, 3, 10, 11 and 15-years, respectively). Compared to other periods, after seven-year storage period, the viability reached a higher value of 94.67%. The correlation coefficient (R^2) between germination percentage and seed viability suggested significant relationship ($R^2 = 0.89$) when seeds were stored for different periods (Table 2).

Seed moisture content

MC recorded for fresh collected seeds (June 2009) was 9.33% (Table 1). This value decreased significantly with storage period to reach 2.83% after 15-years of storage. This parameter had a significant correlation with germination percentage ($R^2 = 0.68$) and seed viability ($R^2 = 0.84$)

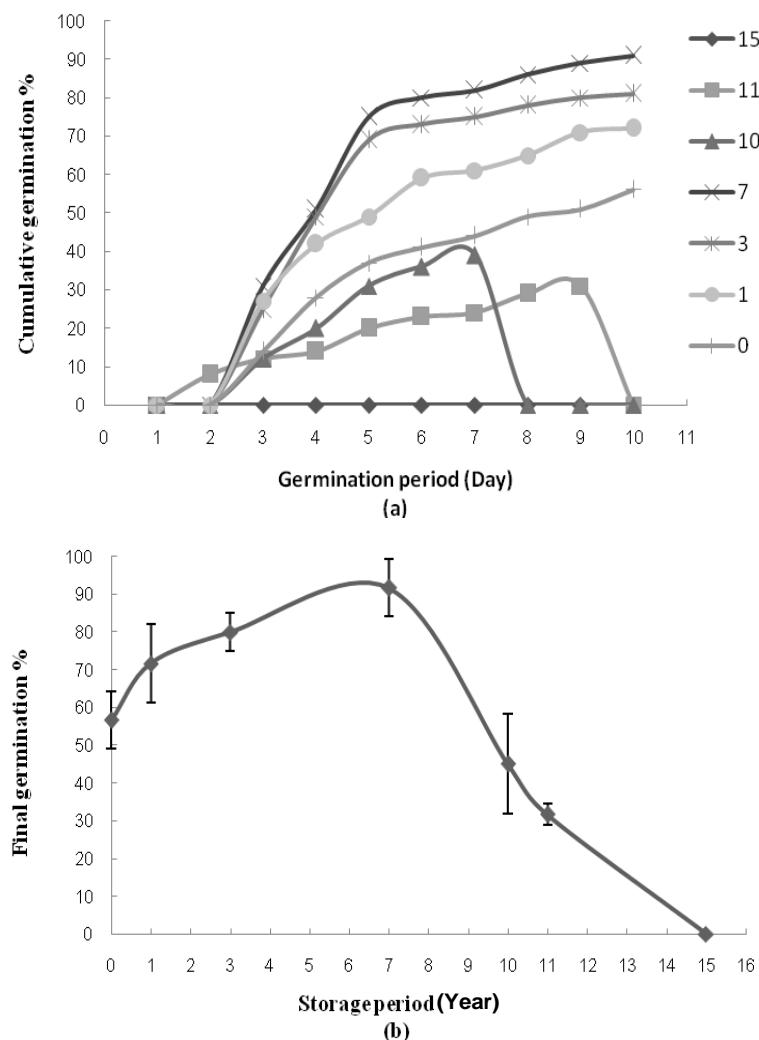


Figure 1. (a) Cumulative germination percentage of *Periploca angustifolia* Labill. seeds stored for different periods. After 15-years of storage seeds germination was completely inhibited ($n = 5$). (b) Survival curve, representing mean final germination, of *Periploca angustifolia* Labill. seeds under long term storage. Values of the final germination percentages (mean, 95% confidence limits, $n=5$), having the same letter are not significantly different ($P>0.05$) (Student-Newman-Keuls' test).

Table 1. Vigor index (VI), viability percentage (V) and moisture content (MC) of *Periploca angustifolia* seeds over the storage period.

Storage period (year)	VI	V	MC
0	42.48 ^c ± 0.11	80.00 ^b ± 4.00	9.33 ^a ± 1.53
1	52.87 ^b ± 2.12	78.67 ^b ± 10.07	9.00 ^a ± 1.00
3	50 ^b ± 1.65	73.33 ^b ± 6.11	7.5 ^{ab} ± 0.50
7	64.26 ± 4.04 ^a	94.67 ^a ± 6.11	7.33 ^{ab} ± 2.08
10	17.13 ± 0.02 ^d	45.33 ^c ± 6.11	5.00 ^{bc} ± 1.00
11	8.64 ± 0.92 ^e	41.33 ^c ± 6.11	4.83 ^{bc} ± 0.76
15	0.00 ^f	24 ^d ± 10.58	2.83 ^c ± 1.04

Means ($n = 5$ for VI, $n = 3$ for V and MC ± SD) within a column followed by the same letter are not significantly different at the 0.05 probability level according to Student-Newman-Keuls test.

Table 2. Correlation coefficients (R^2) between pairs of the studied parameters of *Periploca angustifolia* seeds over the storage period.

Parameter	DPPH	EC	SS	V	MC	PP	GP
EC	-0.33						
SS	0.50	-0.94					
V	0.23	-0.95	0.83				
MC	0.58	-0.80	0.79	0.84			
PP	0.60	-0.79	0.85	0.75	0.80		
GP	0.17	-0.90	0.81	0.89	0.68	0.59	
VI	0.22	-0.93	0.81	0.95	0.78	0.68	0.94

GP, Germination percentage; V, viability percentage; DPPH, radical scavenging activity (%RSA); MC, moisture content; PP, polyphenol content; SS, soluble sugar; EC, electric conductivity; VI, vigor index. Italic values are not significant at $P < 0.05$.

(Table 2). The highest germination and viability percentages were recorded for seeds stored for seven-years, having a moisture content of 7.33%. When MC exceeded this value, as recorded for freshly collected seeds or after one year of storage, the germination percentage decreased suggesting that storability could be improved with MC around 7%.

Seed vigor

EC increased significantly after 10, 11 and 15-years of storage (302, 336 and 404 μSm^{-1} seed $^{-1}$, respectively) showing that seed vigor was drastically affected with storage period (Figure 2a). The lowest EC (197 μSm^{-1} seed $^{-1}$) was recorded for seven-year aged seeds. For the other periods (control, one and three years of storage), the EC were 226.33, 242 and 263.33 μSm^{-1} seed $^{-1}$, respectively. The EC and MC were inversely proportional by over the storage period (Table 1). This indicates that the integrity of the membrane system depends on MC. Within certain limits, MC has negative effects on seed vigor of stored *Periploca* seeds. The correlation coefficients (Table 2) identified between EC and viability, germination percentage and MC suggested significant relationships with $R^2 = -0.95$, -0.90 and -0.80, respectively.

Vigor index

Seven-year aged seeds had significantly higher seed vigor index (64.26) compared with the control and other storage periods (Table 1). The lowest VI was registered for 11-year aged seeds; however, no germination occurred for 15-year old seeds. This parameter was highly correlated with MC, soluble sugars and viability ($R^2 = 0.78$, 0.81 and 0.95) (Table 2). It was negatively correlated with EC ($R^2 = -0.93$). At low MC (<7%), the VI was significantly affected reaching 0 for seeds having 2.83% of MC with no germination. In addition with MC >7%, the VI decreased

significantly reaching 42.48 when MC was 9.3% for freshly collected seeds.

Soluble sugars content

As shown in Figure 2b, seeds stored for 15-years attained lower soluble sugars concentration (6.27 mmol g $^{-1}$ of seed) compared with control and other storage periods. For fresh seeds and those stored for one and seven years, soluble sugars concentration (10.63, 10.54 and 10.34 mmol g $^{-1}$ of seed, respectively) did not show any significant difference. When seeds were stored for three or 10 years, the soluble sugars concentration reached similar values ranging between 9.22 and 9.13 mmol g $^{-1}$ of seed and no significant difference was marked. Correlation analyses revealed positive correlations between soluble sugars concentration and viability ($R^2 = 0.83$), germination percentage ($R^2 = 0.81$) or MC ($R^2 = 0.79$) (Table 2). However, the electric conductivity and the former variable was negatively correlated and suggested significant relationships with ($R^2 = -0.94$).

DPPH and total phenol contents

Seven-year aged seeds showed the lowest antioxidant activity (74.5%) followed by 15 and 11-year old seeds (76.0 and 76.6%, respectively) (Figure 3a). The highest antioxidant activities were recorded for fresh collected seeds and one-year aged seeds (83.8 and 83.2%, respectively). The total phenols varied from 67.3 to 244.7 mg GAE g $^{-1}$ of seed (Figure 3b). The lowest content was registered for aged seeds (15-years) and the highest content was obtained for fresh collected seeds. Regression models were tested to establish relationships between total polyphenol contents and antioxidant activity and the linear regression test show a positive relationship with antioxidant activity and polyphenol contents with $R^2 = 0.60$.

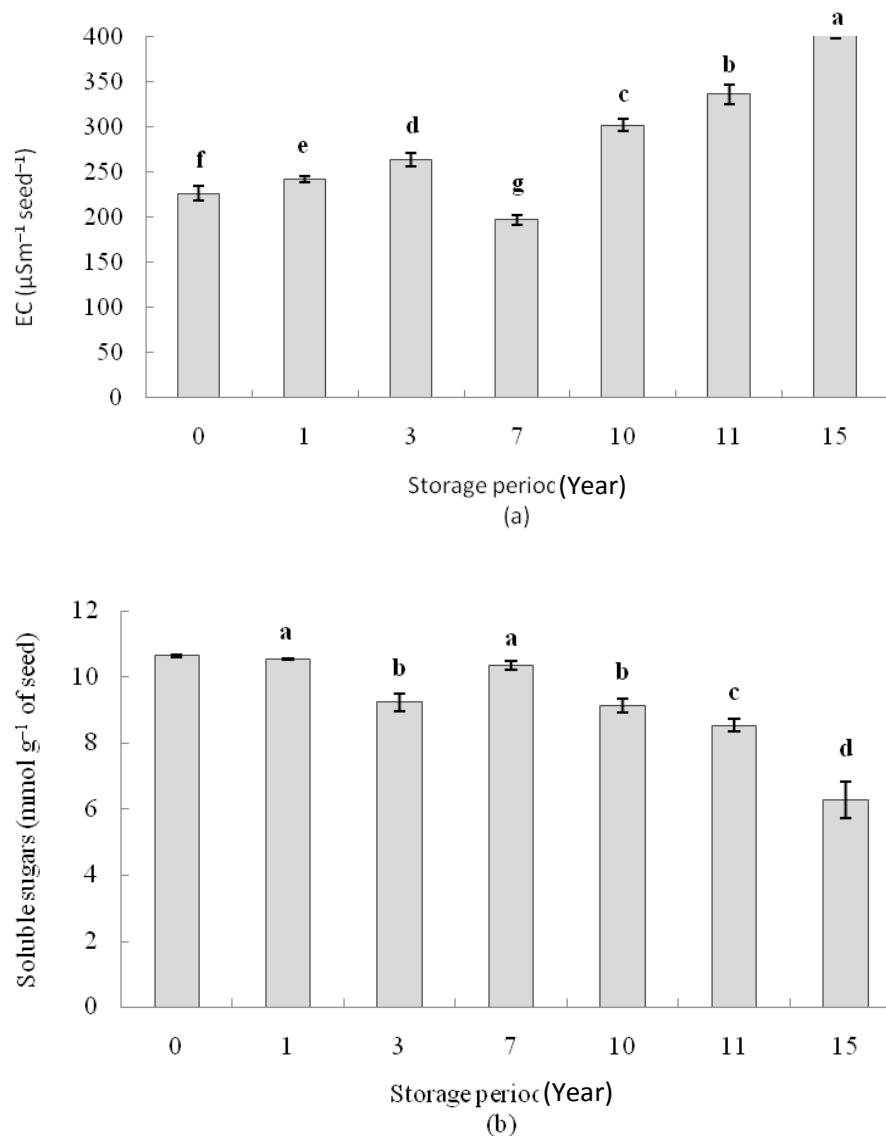


Figure 2. (a) Changes in electric conductivity (μSm^{-1} seed $^{-1}$) and (b) soluble sugar concentrations (mM. g $^{-1}$ seed FW) of *P. angustifolia* seeds over the storage period. Data represent mean \pm SE, $n = 3$.

DISCUSSION

Our results corroborate several other studies and reveal that seed germination characteristics were affected by over storage period (McDonald, 1999; Hsu et al., 2003). Freshly collected seeds presented low percentage of germination however, stored for seven-years they reached its maximum and beyond this period, germination decreased drastically. As indicated by McDonald (1999) and (Hsu et al., 2003), seeds that deteriorated rapidly by increasing storage duration generally showed a marked decline in their ability to germinate. Our data shows that seed viability decreased with storage period except that registered for seven-year old seeds suggesting high

correlation with germination percentage. In aged seeds, because of the destruction of the membrane system, many electrolytes flowed out of cells, so the vigor of seed reduced (Wang et al., 1999). Seed deterioration could be explained by the findings that the ageing of seeds leads to lipid peroxidation that subsequently causes membrane perturbation (Goel and Sheoran, 2003). The change of electric conductivity during seed soaking is commonly used as an indicator for testing the integrity of plasma membrane (Bewley and Black, 1994; Wang et al., 2003). The electric conductivity was therefore evaluated showing significant increase of solute leakage with the storage period that reflects thereby the seed vigor. The lowest electric conductivity was registered for seven-year

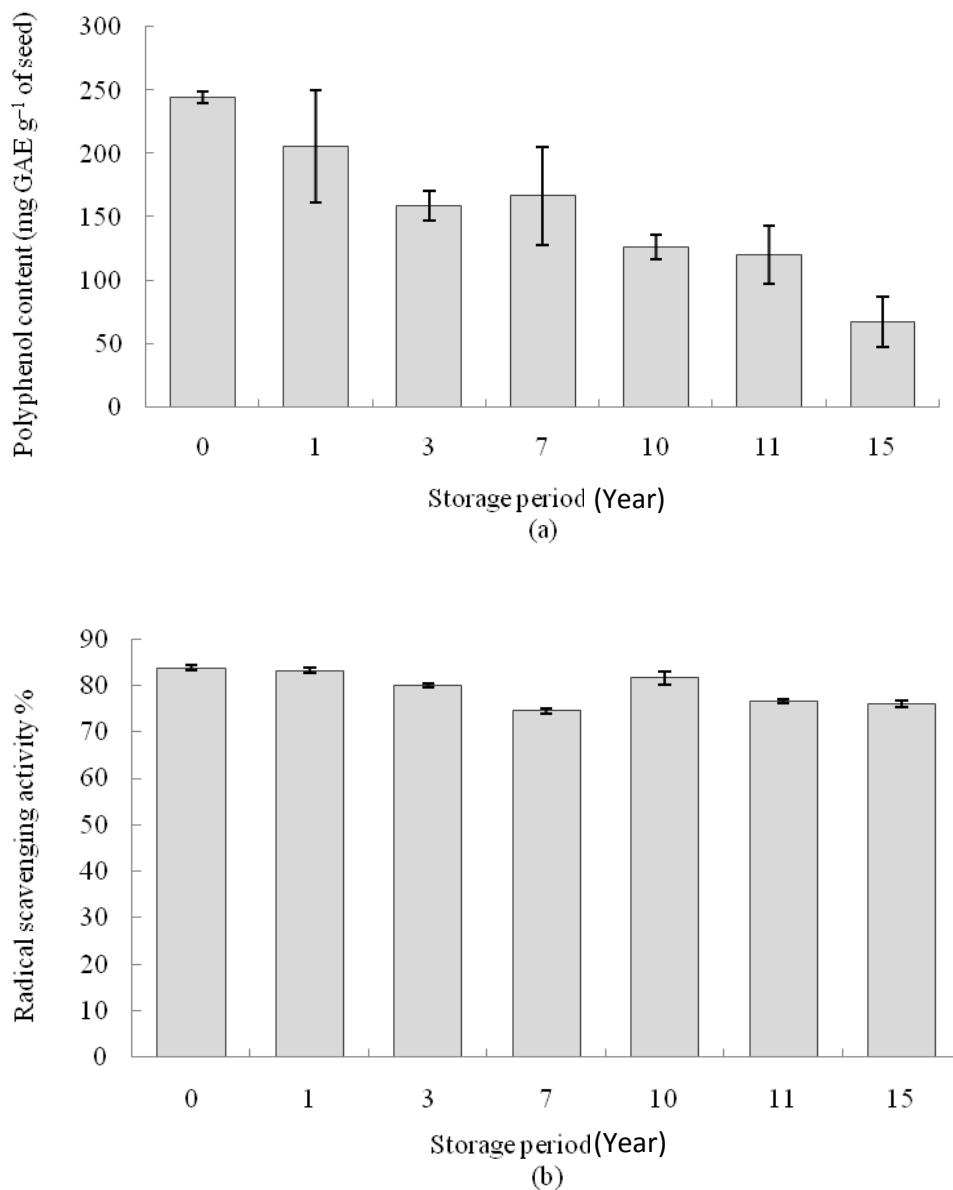


Figure 3. (a) Changes in radical scavenging activity (%RSA) and (b) polyphenol amounts (μg GAE.g⁻¹ of seed FW) of *Periploca angustifolia* seeds over the storage period. Data represents mean \pm SE, $n = 3$.

aged seeds. The data analysis reveals positive correlations between seed vigor, viability and germination percentage. These results prove that germination process is associated with the damage occurring at the membrane as shown by Fujikura and Karssen (1995). McDonald (1999) proved that accelerated aging of seeds is recognized as an accurate indicator of seed vigor and storability. The highest germination percentage, viability and vigor index were registered for seven-year aged seeds having a MC of 7.33%. As proved by Walter et al. (2005), the rate of aging is strongly influenced by environmental and genetic factors such as storage temperature, seed

moisture content and seed quality. Rajjou and Debeaujon (2008) showed that when seeds deteriorate during storage, they lose vigor, become more sensitive to stress during germination and ultimately become unable to germinate. *Periploca* seeds are orthodox type achieving their maturity at low moisture content as many other seeds from the same class (De Tullio and Arrigoni, 2003). They are usually described as dry, although they contain some water, typically in the range of 5 to 10%, depending on the species. Results show that *Periploca* seeds with MC of 7.33% presented the highest germination percentage, viability and seed vigor index. Our approach

provides optimum MC of 7.33% for *Periploca* seeds stored at 20°C. When MC was ≤7%, seed vigor dropped. Analyzing *Zygophyllum xanthoxylon* (Bge.) Maxim seeds, Yi et al. (2008) proved that the best MC maintaining the membrane system was 3.81%. These authors showed that as seed MC declined, the vigor was reduced and the integrity of membrane decreased suggesting that seeds with low MC have shortened longevity. Consequently, low MC storage not only can be used to maintain the quality of seeds but also improve their storability as shown by Wang et al. (2003).

During storage, a large number of reactive oxygen species (ROS) are generated in the seed during ageing which causes lipid peroxidation (McDonald, 1999) and may lead to oxidative stress and cellular damage, resulting in seed deterioration (Rajou and Debeaujon, 2008). However, cells are endowed with detoxifying enzymes and antioxidant compounds that scavenge ROS and play a key role in seed survival, completion of seed germination and seed storability. Substances that could play a role in maintaining seed viability such as phenolic compounds and total soluble sugars were estimated in freshly collected seeds of *Periploca* and those stored from one to 15 years. Seed vigor index showed significant relationships with total soluble sugars ($R^2 = 0.81$) and total phenolic contents ($R^2 = 0.677$). Our results are similar to those reported by Pukacka and Ratajczak (2007) on *Fagus sylvatica* L. seeds stored from two to 10 years showing that germination capacity was strongly and positively correlated with amounts of total phenolic compounds.

During maturity, seeds characteristically accumulate soluble sugars (Amuti and Pollard, 1977). These solutes are known to contribute to the development of tolerance to desiccation and to the longevity itself during storage (Bernal-Lugo and Leopold, 1995; Obendorf, 1997). The protective effect of these solutes is thought to occur through maintaining the structural integrity of membranes and providing stability for macro molecules such as proteins (Lee and Timasheff, 1981; Crowe and Crowe, 1986). Our study shows a high negative correlation between electric conductivity and soluble sugars emphasizing on their role in protecting membranes. Many researchers showed that the presence of these soluble sugars have been found to correlate with longevity (Horbowicz and Obendorf, 1994; Steadman et al., 1996). This fact was also proved in our study, as seed viability was highly correlated with soluble sugars amounts.

In terms of seed germinability, capacity to scavenge ROS seems to be of a particular interest. In our study, fresh seeds showed the highest DPPH scavenging activity percentage, and amounts of polyphenol keeping free radicals and peroxides were at low levels. These findings are aligned with those found by Chang and Sung (1998) and McDonald (1999) suggesting that free radicals and peroxides in freshly collected seeds are generally kept at low levels by cooperative reactions of enzymatic and non-

-enzymatic anti-oxidative systems. Though aged seeds of *Periploca* (15-year old seeds) showed a seed viability of 24%, they did not germinate. During hydration of the aged seed early in germination, catalhydroperoxide lyase becomes active and breaks down oxygenated fatty acids accumulated during storage (Wilson and McDonald, 1986). This reaction may increase the level of free radicals to a point in which the detoxifying capacity of the aged seed has been oversaturated (Gidrol et al., 1994). Therefore, it could well be that seed ageing resulted in an impairment of the enzymatic systems involved in the elimination of the toxic $\cdot\text{O}_2$ intermediate species generated either during dry storage or during germination (Sung and Chiu, 1995; Bailly et al., 1996). This mechanism could explain why intact ungerminated seeds are often metabolically alive, yet the seed dies a day or two after hydration.

P. angustifolia seeds undergo a process of after-ripening under the storage conditions, possibly depending on the low, but steady water loss down to an optimal storage water content of 7.3%, thereafter undergoing some deterioration as indicated by reduced amounts of soluble sugars. When seeds are stored as genetic resources, viability must be maintained for several decades or even centuries. The stringency of the conditions for seed storage increases as the required longevity increases.

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