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Effect of different storage conditions in recalcitrant seeds of holm oak (*Quercus ilex* L.) during germination

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Summary

The effect of three different storage treatments on holm oak (*Quercus ilex* L.) acorns was examined over a period of twelve months. Acorns from two seed lots were stored at 3°C with three different treatments: acorns mixed with peat inside a can with a pierced pipe through the middle, acorns mixed in moistened sand, or acorns inside polyethylene bags without medium. Samples were collected every two months and were analyzed for moisture content, germination and electrolyte leakage. Storage of acorns in polyethylene bags maintained the high seedling percentage usually observed after harvest in germination tests. The acorns stored mixed with peat or sand failed to maintain the germination vigour observed at harvest. Electrolyte leakage was significantly higher for acorns stored in peat rather than for the other two treatments. However, acorns stored in sand and in peat had a higher level of moisture content than those kept in polyethylene bags. These results suggest that storage of acorns from the recalcitrant holm oak in polyethylene bags could preserve the acorn germination over a longer period of time with respect to the usual methods.

Introduction

Seed recalcitrance has been studied for nearly forty years (Roberts, 1973; Bonner and Vozzo, 1987; Bonner, 1996; Finch-Savage *et al.*, 1996; Suszka *et al.*, 1996; Suszka *et al.*, 1976). Storage of recalcitrant seeds have also been investigated recently (Ozbingol and O'Reilly, 2005), since up to date the storage conditions for recalcitrant seeds are not yet fully elucidated.

Recalcitrant seeds have a relatively high water content at the maturation stage (Berjak and Pammenter, 2000), difficult post-harvest handling due to degradation phenomena, and no possibility for reducing the seed water content below a stated level (Bonner, 2003). For this kind of seeds, there are only few studies, which state storage periods longer than a couple of years (Suszka *et al.*, 1996). In contrast, the so-called orthodox seeds are characterized by moisture content below or up to 12% and are easy to manage and store for a long period (Connor and Sowa, 2002 and 2003).

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The utilization of recalcitrant seeds has some problems linked to the following aspects. Firstly, the freshly harvested seeds are not deeply dormant, often being subjected to pre-germination (Farnsworth, 2000), a phenomenon which may start even prior to seed dispersal and it can last until a couple of months after the harvest. This means that seeds are sprouting, becoming more vulnerable to pathogens. In addition, they are much more delicate to handle, as the seeds themselves are strongly compromised due to damage of the primary radicle. Secondly, recalcitrance often prevents the storage of seed lots for more than six months, as after this period the seeds become dry and they lose their viability; these seeds hence are available only within a few months after the harvest. Thirdly, the early decrease in germination in recalcitrant seeds could involve the deterioration of the cell membranes (Connor and Sowa, 2002 and 2003), because loss of seed vigour is related to a high ion leakage (Fang-yuan *et al.*, 2006). Fourthly, some recalcitrant species, in particular white oaks, can show different levels of epicotyl dormancy, due not only to biochemical processes, but also to the interspecific and intraspecific differences related to the latitude of a particular geographical area (Ghasemi and Khosh-Khui, 2007). In particular, acorns can show delayed germination immediately after harvest, but this can be overcome with the stratification of the acorns. During stratification, lipids, proteins and nucleic acids undergo mobilization from one region of the acorns to another as they pass from dormancy to germination (Bonner and Vozzo, 1987).

In 2000 Farnsworth presented a list of the main plant species with recalcitrant seeds, which includes the *Quercus* genus. Among European species, holm oak (*Q. ilex* L.), a monoecious and a highly self-incompatible species (Yacine *et al.*, 1996), is native to several areas of the Mediterranean basin (Reyes and Casal, 2006). This species is readily available and with a rather high seed moisture optimum (approx. 40%) at harvest time (Monteleone *et al.*, 2001), which is comparable to that of the other species of genus *Quercus* (Bonner and Vozzo, 1987). In contrast, in Italy holm oak is the easiest to handle according to practical experience, because its acorns remain protected from mould attack for at least six months after harvest. This feature should allow a "slower" deterioration of the seed and an easier identification of factors affecting it. In addition, while *Q. robur* and *Q. petraea* can be stored up to three years at -3°C (Suszka *et al.*, 1996), holm oak acorns are sensitive to mild freezing storage conditions (Monteleone *et al.*, 2001) and are not able to maintain germination for more than one year.

For these reasons, in this research holm oak has been chosen as a model recalcitrant species, which is particularly hard to store, with the aim to verify the efficacy of some storage treatments on the survival and vigour of acorns from holm oak and to evaluate how seed water content and leakage of electrolytes affected their germination.

Materials and methods

Plant material and preliminary treatments

Two acorn seed lots of holm oak, both harvested at the end of November 2008, were collected from two distinct stations of the Po Valley: Monte Luppia near Garda Lake (VR) (CE/IT/320/qil/1/VR/137, 45°34'45" N, 10°40'46" E) [seed lot 164/08] and Porto Caleri

(RO) (CE/IT/430/qil/1/RO/101, 45°06'21" N, 12°19'44" E) [seed lot 211/08]. Acorns were collected from ten different trees. Collection of the acorns was done by shaking the tree at full seed maturity, according to the indexes of maturity described by Bonner (2003) and Monteleone *et al.* (2001). The indexes used were the following: the natural occurrence of a slight fall; a green-browning of the epicarp; an easy separation of acorn from the cupule; a light colour of the scar after removal of the cupule; a white-clear colour of cotyledons. The collection of acorns was performed after approximately 10% natural shedding to avoid their possible degradation by fungi, frequently observed during fruit permanence on the ground. The acorns were collected randomly, without any physical selection, thus representing all the variability present in the mother trees.

The collected acorns were placed in plastic boxes and were subjected, within 48 hours from the harvest time, to manual selection. Before the selection, the acorns were stored in a cold room at +3°C (Gosling, 1989). During the selection all the impurities such as twigs, leaves, stones or soil were removed according to Bonner procedure for cleaning (2003), and afterwards the cupules were easily removed by hand. All the acorns that seemed, on visual inspection, to be damaged by insects or fungi were removed, as well as immature acorns, according to the previously described indexes of maturity (Bonner, 2003). At the end of the process, two batches of clean seeds were obtained, each batch weighing 20 kg. The total number of seeds for each seed lot was higher than the scheduled seeds in the test plan, so that at the end of selection process damaged acorns were replaced by healthy ones, without decreasing the exact seed number needed for the experiments.

Batches were subjected to thermotherapy, in order to eliminate or reduce potential outbreaks of pathogens during storage in the refrigerator cell. To this purpose, seeds were immersed into water at 41°C for a period of 2.5 h (Suszka *et al.*, 1996). During the water immersion, the floating acorns were replaced with others, as such behaviour is characteristic of malformed (abnormal) or insect damaged seeds (Knudsen *et al.*, 2004). After the thermotherapy, the acorns were treated by soaking with a fungicide solution for 10 min [ROVRAL FL, 1 g a.p. iprodione kg^{-1} seed, i.e. 134 ml of active ingredient for 1 L of water + 20 ml Etravon (adhesivant)] to reduce fungal attacks. Thermotherapy has been demonstrated to be applied to *Quercus* spp. (Finch-Savage *et al.*, 2003; Belletti *et al.*, 2001), without any alterations on subsequent seed viability.

The treated acorns were dried by air circulation at room temperature for 0.5 h. This treatment aimed to remove the water present on the surface of the epicarp, without affecting the moisture of the whole acorns (cotyledons together with embryos) measured at the harvest time (results not shown).

Quality screening test

The dried acorns were subjected to an initial quality screening immediately after harvest. The analysis was performed on two seed lots, each containing 600 seeds.

To define the acorns' quality, the following characteristics, listed below, were considered: i) cut test; ii) moisture content; iii) 1000 seed weight; iv) germination and v) leakage. Some of these measurements (moisture, germination and leakage) were also performed every two months throughout the whole period of storage. The quality of the two seed lots at the harvest stage (November 2008) is shown in table 1.

The *cut test* was performed according to the procedure reported in Suszka *et al.* (1996) on four replicates of 50 seeds each.

The *moisture content* was measured according to the ISTA low-temperature oven method, putting 8 replicates, each containing 4.5 ± 0.5 g of seeds cut in pieces, into a forced ventilated oven at 103-107°C for 17 ± 1 h (ISTA Rules, 2008). The data are expressed, as a percentage, on the fresh weight basis.

The *weight of 1000 seeds* was determined as the mean of eight replicates, each containing 100 seeds, chosen randomly in the lot of 20 kg (ISTA Rules, 2008). The value was expressed in g per fresh weight.

Table 1. Seed quality screening tests of seed lots 164/08 and 211/08, each containing 600 acorns evaluated at harvest time (November 2008).

Seed lot	Cut test (%)	Moisture content (%)	1000 seed weight (g)	Germination (%)	Conductivity (μS g^{-1})
164/08	91 ± 1.5	40.2 ± 0.1	3111.1 ± 97.2	68 ± 10	0.4 ± 0.1
211/08	86 ± 1.5	40.7 ± 0.1	2850.1 ± 143.4	63 ± 10	1.5 ± 0.1

Leakage of electrolytes

Electrolyte leakage was carried out according to the method reported by Bonner (1996), with minor changes. Briefly, a sample of 20 seeds was weighed and soaked in 200 ml of ultrapure water and maintained in a closed container for 48 h at 20°C. After removing the seeds for the germination test below described, the conductivity of the solution was measured with a Crison conductance-meter (model EC-BASIC 30). The conductivity of the blank (distilled water) was subtracted from each sample reading, and the result was divided by the initial weight of the seeds. Conductivities were expressed as μS g^{-1} of fresh weight. Data are mean of 8 sample replicates consisting of 20 seeds each. This parameter represented the amount of solutes released in the solution, after seed imbibition. Membrane damage and dead tissue are associated with very high conductivity values.

Germination test

An assay was performed on the same seeds used to measure electrolyte leakage. Eight replicates of 20 seeds of each seed lot were made, according to ISTA Rules (2008). Acorns were placed in standard controlled conditions (20°C, 70% relative humidity, and 24 h in the dark) for a total period of 28 days. The germination was expressed as the ratio between the number of normal seedlings, with a hypocotyl equal or more than the length of the whole acorn emerged within the period specified, and the total amount of acorns considered. There were also a considerable number of pre-germinated acorns: the acorns were counted as pre-germinated when the hypocotyls had already sprouted on the first day of the test. Pre-germinated seeds were included in the total amount of germinated acorns.

Seed storage treatments

An experimental plan was assessed on the basis of preliminary data collected, in order to detect the natural deterioration of the seeds tested. Three different storage treatments were assayed: i) plastic can with peat; ii) stratification on sand; iii) polyethylene (PE) bags without any medium.

All storage treatments were maintained in a cold room at 3°C, in the dark with 60% air moisture. Mild freezing temperatures (lower than -3°C), applied during acorn conservation, have been suggested by some authors (Suszka *et al.*, 1996; Kehr and Schroeder, 1996), but they represent a more expensive procedure and extensive cold stores are required.

Plastic can with peat. The classic method of storage for acorns, according to Suszka *et al.* (1996), was applied, except for the storage temperature. The seeds were mixed with dry peat (approx. moisture content 35%) and then placed in two cans with a volume of 20 litres (each for one seed lot). In these cans, a central perforated pipe allowed air circulation from the bottom to the top of the can. On the lid of the can there was a disc of cardboard and the lid was placed on the bin without screwing it on.

Sand stratification is a method used in nurseries. The seeds were stratified in sieved sand (i.e. a layer of sand and a layer of seed) and placed into two open plastic basins with a volume of 40 l, each for one seed lot. The sand was then moistened and kept at the saturation point for all the duration of the test.

Polyethylene bag is an alternative method, fairly common in literature (Rink and Williams, 1984; Gosling, 1989; Bonner, 2003) for the seed storage as it stands. For each sampling date and seed lot, two transparent, waterproof bags of 0.03 mm of thickness were prepared. Each bag (volume of 5 l) contained 100 seeds and was tightly closed. After each bag was opened, the seeds were rapidly tested.

Experimental plan

Starting from December 2008 to October 2009, samples of 200 stored acorns were collected at six sampling dates, each every two months, and immediately analyzed for moisture content, electrolyte leakage and germination.

Statistical analysis

In all experiments, moisture, germination and electrolyte leakage data were evaluated by factorial ANOVA with the software STATISTICA package. Statistical analysis was performed on samples, each containing 200 acorns, using 8 replicates for each test assay. Means were compared by the Scheffé's test. In the case of data expressed as a percentage (moisture content and germination test), arcsin $(\text{percentage}/100)^{1/2}$ transformation of the variable was performed.

Results

On the basis of preliminary assays, the seed lots selected exhibited a medium-high quality. There were small differences in the quality of the lots: the most marked was seen in the leakage of electrolytes (table 1). Since the climatic conditions may influence plant water status, an analysis of rainfall and temperature patterns in the different stations was carried out (during harvest season 2008), starting from flowering to acorn maturation (data not shown). Monte Luppia station underwent less severe water deprivation than Porto Caleri; in particular holm oaks were exposed to two prolonged critical periods of water stress in the case of Porto Caleri, while the plants located on Monte Luppia were affected by mild stress for a shorter time.

Moisture content

Both seed lots, at the end of November 2008 (harvest stage), exhibited the same moisture content (figures 1A and 1B), i.e. approx. 40%. During the storage period, acorn moisture content increased significantly in the sand treatment ($P < 0.05$), reaching about 50% in both seed lots. The other treatments had similar response, even though there was a slight higher increase in the moisture content of seed lot 211/08.

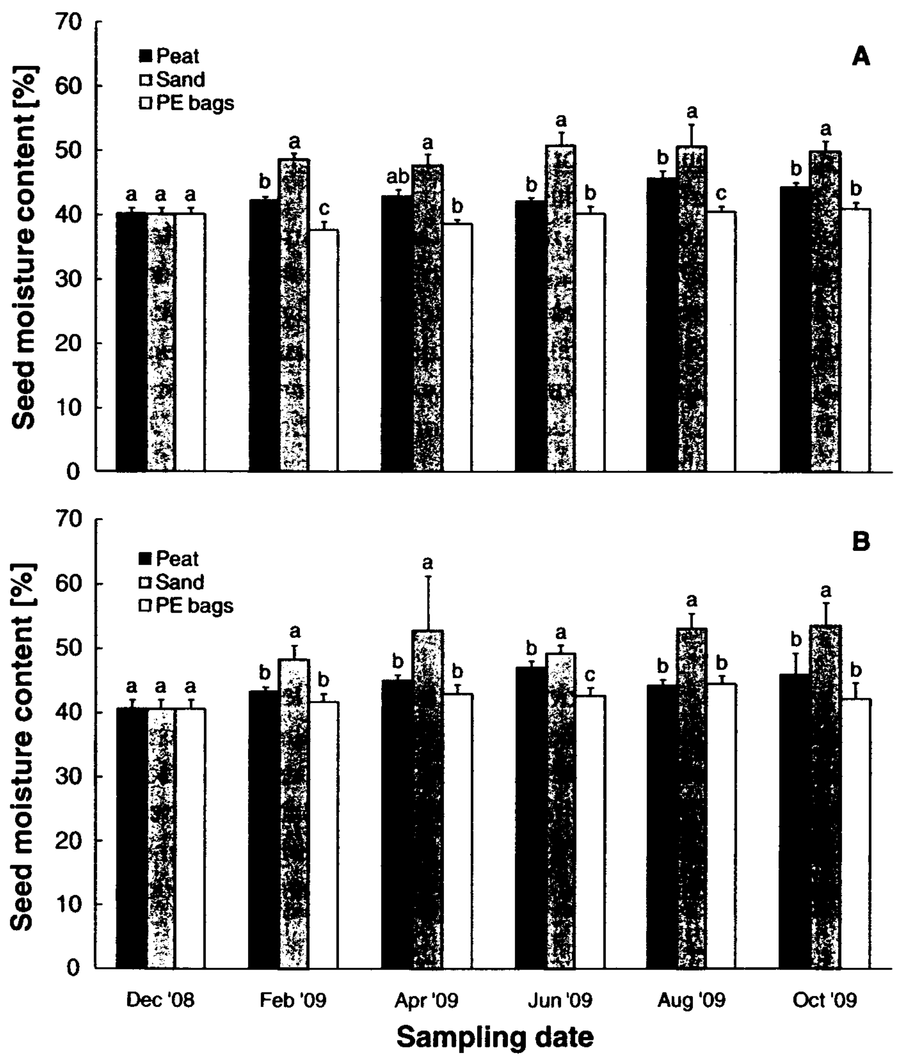

Figure 1. Effect of different storage treatments on seed moisture content during different sampling dates in holm oak. Acorns from seed lot 164/08 (*A*) and 211/08 (*B*) were stored in plastic can with peat (■), in sand (■), or in plastic bags (□). Values represent the mean (±SD) of at least eight independent replicates. Data are calculated after arcsine transformation and changed back to the original scale. Means with different letters are significantly different at $P \leq 0.05$, as determined by Scheffé's test.

All the factors and their interactions were strongly significant, as shown in table 2, with the exception of the interaction Seed lot × Treatment, indicating that treatment effects were similar across both seed lots.

Table 2. ANOVA results showing the effect of sampling date, seed lot and storage treatment on acorn moisture, electrolyte leakage and germination in holm oak. Sampling Date = D; Treatment = T; Seed lot = S.

Variation source	Df	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
		Moisture			Electrolyte leakage			Germination		
D	5	0.024	50.9	**0.0000**	138.849	151.7	**0.0000**	3.548	153.4	**0.0000**
S	1	0.029	61.3	**0.0000**	84.310	92.1	**0.0000**	0.073	3.2	0.0760
T	2	0.152	322.6	**0.0000**	551.885	603.2	**0.0000**	2.516	108.8	**0.0000**
D × S	5	0.002	3.4	**0.0055**	6.755	7.4	**0.0000**	0.117	5.1	**0.0002**
D × T	10	0.006	13.3	**0.0000**	51.364	56.1	**0.0000**	0.397	17.3	**0.0000**
S × T	2	0.001	2.7	0.0679	0.448	0.5	0.6133	0.041	1.8	0.1734
D × S × T	10	0.002	4.2	**0.0000**	4.835	5.3	**0.0000**	0.044	1.9	**0.0467**
Error	252	0.000			0.915			0.023		

Germination

Germination increased up to four months of storage in both seed lots, as expected for this species, which has, as already known, weak dormancy (figures 2A and 2B). This effect is independent from treatment and all the samples exhibited the same pattern. In the case of the acorns stored in peat and sand, the germination increase included a substantial proportion of pre-germinated seeds. In particular, after four months of storage in peat (April 2009), the pre-germinated seeds reached values of 63 and 69% for seed lots 164/08 and 211/08, respectively. Similarly, sand treatment showed 49 and 66% of pre-germinated seeds for the seed lots from Monte Luppia and Porto Caleri, respectively. This is an unfavourable feature, because it lowers the seed quality for forest nursery purpose. Indeed, starting from June 2009, there was a steep decline in seed germination for both acorns stored in peat and sand.

In contrast, the PE bag storage preserved the seed germination from early radicle emergence, and the acorns stored in PE bags maintained more than 50% germination even though it decreased slightly. This pattern could be observed in both seed lots as confirmed by ANOVA test (table 2).

Electrolyte leakage

Storage of acorns mixed with peat resulted in a greater increase in electrolyte leakage from the acorns, when compared with the other treatments (figures 3A and 3B). Such an increase occurred early after harvest and proceeded progressively during the whole storage period, reaching a final value that was five-fold higher than that initial. The other treatments showed similar slight variations in leakage at the different sampling dates, which were significantly lower than those measured in acorns stored in a plastic can with peat ($P < 0.05$, as determined by Scheffè's test in table 2).

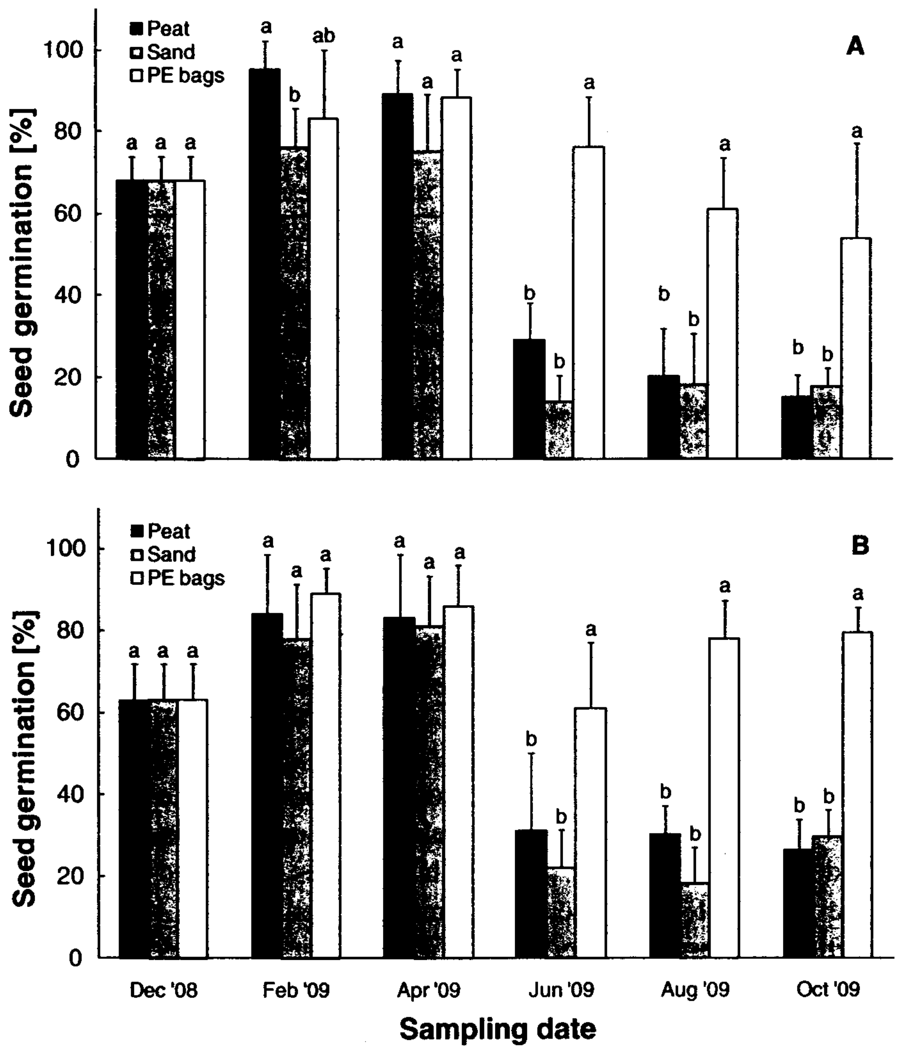

Figure 2. Effect of different storage treatment on seed germination during different sampling dates in holm oak. Acorns from seed lot 164/08 (*A*) and 211/08 (*B*) were stored in plastic can with peat (■), in sand (▩), or in plastic bags (□). Values represent the mean (±SD) of at least eight independent replicates. Data are calculated after arcsine transformation and changed back to the original scale. Means with different letters are significantly different at $P \leq 0.05$, as determined by Scheffé's test.

Although the two seed lots showed different levels of electrolyte leakage from the acorns, at the first sampling date (December 2008) and throughout the whole storage period, this parameter was similarly affected by treatments, independently from seed provenance. In fact, as shown in table 2, the interaction Seed lot × Treatment was not significant, while all the other factors and interactions were highly significant ($P < 0.001$).

The other experimental factors, such as Sampling date and Treatment, showed a very high significance ($P < 0.001$), similarly to the interactions Sampling date × Seed lot and Sampling date × Treatment ($P < 0.001$), while at a lower extent, also Sampling date × Seed lot × Treatment ($P < 0.05$) caused a significant difference in the statistical analysis of the data.

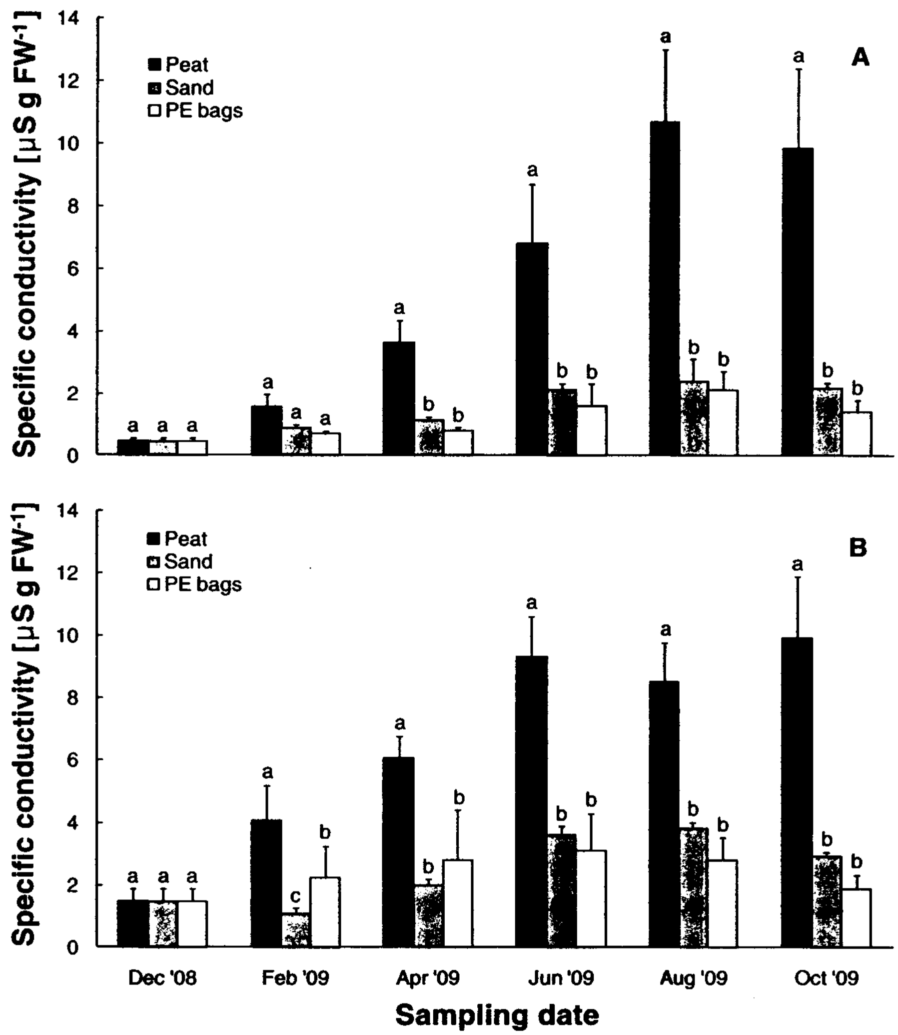

Figure 3. Effect of different storage treatment on seed electrolyte leakage during different sampling dates in holm oak. Acorns from seed lot 164/08 (*A*) and 211/08 (*B*) were stored in plastic can with peat (■), in sand (▣), or in plastic bags (□). Values represent the mean (±SD) of at least eight independent replicates. Means with different letters are significantly different at $P \leq 0.05$, as determined by Scheffé's test.

Discussion

The results here described confirm that a recalcitrant species, such as holm oak, was difficult to store longer than few months. In particular, the usual storage techniques (plastic can with peat and wet sand stratification) did not maintain the germination during the overall experimental period, showing a sudden decrease in this parameter after about 6 months. This observation could be applied to both seed lots, demonstrating that it is a general feature, despite the geographical origin (Rink and William, 1984; Bonner, 1996; Connor and Sowa, 2002 and 2003; Knudsen *et al.*, 2004). On the contrary, the storage inside PE bags prolonged the shelf-life of the acorns significantly, maintaining adequate germination vigour even after 11 months of storage. This effect could be due to both the steady level in moisture content and the mild increase in electrolyte leakage showed by PE bag treated acorns.

Both wet sand and PE bag treatments induced a moderate increase in electrolyte leakage during storage, which may be indicative of physiological changes linked to the onset of germination. In fact, as discussed by Connor (2004), recalcitrant seeds, which cannot be dried before conservation, continue to respire even during low temperature storage and, thus, they can pre-germinate. On the contrary, peat treatment caused a dramatic rise (more than five-fold with respect to the other treatments) in electrolyte leakage, suggesting that in this case the event could be ascribed to an irreversible loss of membrane integrity rather than to a rise of normal metabolic activities. In agreement, several papers show a positive correlation between decline in germination capacity and electrolyte leakage from seeds (Becwar *et al.*, 1982; Pukacka and Ratajczak, 2006; Panza *et al.*, 2007).

Such membrane damage could be due to an enhanced oxidative stress, not counteracted by an adequate antioxidant system, and could be associated to a high pre-germination rate (Oracz *et al.*, 2009). However, a direct correlation between pre-germination and high conductivity could not be found, since sand stratification treatment exhibited a high pre-germination level without any significant variation in electrolyte leakage.

Two seed lots from different geographical areas were analyzed, with the aim to compare the effect of storage conditions on acorn germination. A storage temperature of 3°C, chosen for all treatments, was applied according to Ghasemi and Khosh-Khui (2007), even though a mild freezing temperature (-2 or -3°C) was suggested by Suszka *et al.* (1996). This statement was based on experimental data obtained in species grown in Poland, i.e. in species adapted to environmental conditions typical of continental climate. Since holm oak is a Mediterranean species, mild freezing conditions might compromise embryo viability, as confirmed by Monteleone *et al.* (2001). These authors demonstrated that prolonged conditioning at -5°C of acorns from holm oak grown in Italy resulted in a lower germination rate, when compared to storage at 0°C. For this reason, all the storage treatments were performed above freezing point. Furthermore, thermotherapy and fungicide-treatment replaced the mould-preventing effect of the mild freezing temperature.

Fresh plant material, at harvest stage, showed similar quality parameters, except for the electrolyte leakage. In the seed lot 211/08, a relatively high conductivity, when referred to fresh seeds, may be explained by the different geographical area of provenance, characterized by different climate parameters. According to climatic data analysis, seed lot 211/08 underwent a longer and more severe water deprivation during its maturation, when compared to seed lot 164/08. Hence, the water status might explain the difference in weight and electrolyte leakage observed in the two seed lots at the harvest (table 1). On the other hand, it is unlikely that these variations are due to genetic features, due to the lack of specific ecotypes in holm oak populations in the Northern Italy.

Climate conditions are one of the major factors influencing the acorn production in holm oak, as shown by Carevic *et al.* (2009). In particular, water deprivation could impair membrane integrity during fruit maturation and this alteration could explain the initial variability in electrolyte leakage observed in the two seed lots. Actually, this phenomenon did not affect the response to storage treatments, as shown by the lack of significance in the interaction Seed lot × Treatment in all the investigated parameters (see table 2). Nevertheless, acorns of different seed lots exhibited different leakage patterns during storage in response to treatments, as stated by three way interaction analysis (table 2), indicating that, at least in some sampling dates, acorn provenance was a discriminating factor.

An increase of moisture content in wet sand treatment was observed starting from 2 months after the harvest (February 2009), probably caused by the continuous watering of the substrate. In addition, acorn integument can be affected by prolonged high moist conditions. Since Pritchard and Manger (1990) suggested that regulation of the dormancy process is linked to pericarp, its deterioration may lead to premature seed imbibition and the consequent unfavourable pre-germination.

It is suggested that prolonged storability implies the maintenance, as long as possible, of the initial levels in both electrolyte leakage and moisture content. The fulfilment of these conditions could be a useful tool to predict the effect of a storage treatment on the decrease in germination level. On the contrary, the measurement of a single parameter during storage could not be exhaustive for the indication of the seed vigour in holm oak.

The modified atmosphere inside the PE bag prevented the seed from both water absorption and the consequent pre-germination (Finch-Savage, 1992; Finch-Savage and Clay, 1994a), or desiccation leading to loss of the germination vigour (Gosling, 1989). The PE bag effects confirmed observations from similar experiments performed on *Quercus* spp. acorns (Rink and Williams, 1994; Ozbingol and O'Reilly, 2005), where an adequate germination rate was preserved during time by storage inside plastic bags. In addition, Suszka *et al.* (1996) stated that modified environment (high CO_2 and low O_2 concentration) could be responsible for the maintenance of germination in *Quercus cerris* L.. This was a further critical parameter for the treatments with medium (plastic can with peat and sand stratification), since they did not allow an atmosphere modification during acorn storage.

Plastic film can also allow the accumulation of ethylene, probably released by seeds, which could exert a hormonal effect. With regard to this phenomenon, there is conflicting evidence, since it is known that ethylene promotes orthodox seed germination by dormancy breaking (Lalonde and Saini, 1992; Farnsworth, 2000); on the other hand, it is argued that the hormone inhibits germination in *Quercus robur* L., a recalcitrant species like holm oak (Finch-Savage and Clay, 1994b).

These topics need to be investigated further with a biochemical approach, involving the analysis of gas fluctuation inside the storage environments and the oxidative/antioxidative activities in the seed embryo. Therefore, further work is needed to clarify the physiological processes involved in germination maintenance during acorn storage in PE bags. In particular, it appears to be crucial to investigate if this storage treatment is linked to an increased CO_2/O_2 ratio, which could minimize respiration and oxidative processes, affecting energetic metabolism.

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References

Becwar, M.R., Stanwood, P.C. and Roos, E.E. (1982). Dehydration effects on imbibitional leakage from desiccation-sensitive seeds. *Plant Physiology*, **69**, 1132-1135.

Berjak, P. and Pammenter, N.W. (2000). Aspects of recalcitrant seed physiology. *Revista Brasileira de Fisiologia Vegetal*, **12**, 56-69.

Belletti, P., Monteleone, I. and Cartarasa, M. (2001). Optimisation of stone-oak seed management aimed at nursery production of high quality seedlings for reforestation. In *Proceedings International Conference "Forest research: a challenge for an integrated European approach". Thessaloniki, August 27 – September 1*, pp. 413-416.

Bonner, F.T. and Vozzo, J.A. (1987). Seed biology and technology of *Quercus*. USDA, Forest service, *General Technical Report*, GTR SO-66, New Orleans, LA.

Bonner, F.T. (1996). Responses to drying of recalcitrant seeds of *Quercus nigra* L. *Annals of Botany*, **78**, 181-187.

Bonner, F.T. (2003). Collection and care of acorns. A practical guide for seed collectors and nursery managers-version 1.1.

Carevic, F.S., Fernández, M., Alejano, R., Vázquez-Piqué, J., Tapias, R., Corral, E. and Domingo, J. (2009). Plant water relations and edaphoclimatic conditions affecting acorn production in a holm oak (*Quercus ilex* L. ssp. ballota) open woodland. *Agroforestry Systems*, **78**, 299-308.

Connor, K.F. and Sowa, S. (2002). Recalcitrant behaviour of temperate forest tree seeds: storage, biochemistry and physiology. Proceedings of the eleventh biennial southern silvicultural research conference, *General Technical Report*, SRS-48.

Connor, K.F. and Sowa, S. (2003). Effects of desiccation on the physiology and biochemistry of *Quercus alba* acorns. *Tree Physiology*, **23**, 1147-1152.

Connor, K.F. (2004). An update on oak seed quality research: hardwood recalcitrant seeds. In *National Proceedings, forestry and conservation nursery associations*-2003. *Ogden (UT): USDA Forest Service, Rocky Mountain Research Station.* RMRS-P-33, pp. 111-116.

Fang-yuan, Y., Yan, D. and Shen, Y. (2006). Physiological characteristics changes of *Aesculus chinensis* seeds during natural dehydration. *Journal of Forestry Research*, **17**, 103-106.

Farnsworth, E. (2000). The ecology and physiology of viviparous and recalcitrant seeds. *Annual Reviews of Ecology and Systematics*, **31**, 107-138.

Finch-Savage, W.E. (1992). Embryo water status and survival in the recalcitrant species *Quercus robur* L.: evidence for a critical moisture content. *Journal of Experimental Botany*, **43**, 663-669.

Finch-Savage, W.E. and Clay, H.A. (1994a). Water relations of germination in the recalcitrant seeds of *Quercus robur* L.. *Seed Science and Research*, **4**, 315-322.

Finch-Savage, W.E. and Clay, H.A. (1994b). Evidence that ethylene, light and abscisic acid interact to inhibit germination in the recalcitrant seeds of *Quercus robur* L.. *Journal of Experimental Botany*, **45**, 1295-1299.

Finch-Savage, W.E., Blake, P.S. and Clay, H.A. (1996). Desiccation stress in recalcitrant *Quercus robur* L. seeds results in lipid peroxidation and increased synthesis of jasmonates and abscisic acid. *Journal of Experimental Botany*, **47**, 661-667.

Finch-Savage, W.E., Clay, H.A., Budge, S.P., Dent, K.C., Clarkson J.P. and Whipps, J.M. (2003). Biological control of *Sclerotinia pseudotuberosa* and other fungi during moist storage of *Quercus robur* seeds. *European Journal of Plant Pathology,* **109**, 615-624.

Ghasemi, M. and Khosh-Khui, M. (2007). Effects of stratification and growth regulators on seed germination and seedling growth of *Quercus ilex* L.. *Journal of Plant Science*, **2**, 341-346.

Gosling, P.G. (1989). The effect of drying *Quercus robur* acorns to different moisture content, followed by storage, either with or without imbibition. *Forestry*, **62**, 41-50.

International seed testing association (2008). International rules for seed testing. Chapter 5 "Germination", adopted at the Ordinary Meeting 2007, Iguaçu Falls, Brazil. V 1 - V 46.

International seed testing association (2008). International rules for seed testing. Chapter 9 "Determination of Moisture Content", adopted at the Ordinary Meeting 2007, Iguaçu Falls, Brazil. IX 1 - IX 20.

Kehr, R.D. and Schroeder, T. (1996). Long-term storage of oak seeds – new methods and mycological aspects. In *Proceedings of the Tree Pathology Meeting, Opocno, Czech Republic, October*. pp. 50–61.

Knudsen, I.M.B., Thomsen, K.A., Jensen, B. and Poulsen, K.M. (2004). Effects of hot water treatment, biocontrol agents, disinfectants and a fungicide on storability of English oak acorns and control of the pathogen, *Ciboria batschiana. Forestry Pathology*, **34**, 47-64.

Lalonde, J. and Saini, H.S. (1992). Comparative requirement for endogenous ethylene during seed germination. *Annals of Botany*, **69**, 423-428.

Monteleone, I., Cartarasa, M. and Belletti, P. (2001). Aspetti sementieri e vivaistici del leccio. *Sherwood, Foreste ed alberi oggi,* **7**, 35-39.

Oracz, K., El-Maarouf-Bouteau, H., Kranner, I., Bogatek, R., Corbineau, F. and Bailly, C. (2009). The mechanisms involved in seed dormancy allevation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signalling during germination. *Plant Physiology,* **150**, 494-505.

Ozbingol, N. and O'Reilly, C. (2005). Increasing acorn moisture content followed by freezing-storage enhances germination in pedunculate oak. *Forestry*, **78**, 73-81.

Panza, V., Láinez, V., Maldonado, S. and Maroder, H. (2007). Effects of desiccation on *Euterpe edulis* Martius seeds. *Biocell*, **31**, 383-390.

Pritchard, H.W. and Manger, K.R. (1990). Quantal response of fruit and seed germination rate in *Quercus robur* L. and *Castanea sativa* Mill. to constant temperatures and photon dose. *Journal of Experimental Botany*, **41**, 1549-1557.

Pukacka, S. and Ratajczak, E. (2006). Antioxidative response of ascorbate–glutathione pathway enzymes and metabolites to desiccation of recalcitrant *Acer saccharinum* seeds. *Journal of Plant Physiology*, **163**, 1259-1266.

Rink, G. and Williams, R.D. (1984). Storage technique affects white oak acorn viability. *Tree Planters' Notes*, **35**, 3-5.

Reyes, O. and Casal, M. (2006). Seed germination of *Quercus robur*, *Q. pyrenaica* and *Q. ilex* and the effect of smoke, heat, ash and charcoal. *Annals of Forestry Science*, **63**, 205-212.

Roberts, E.H. (1973). Predicting the storage life of seeds. *Seed Science and Technology*, **1**, 499-514.

Suszka, B. (1976). Studies on the long-term storage of acorns. Polish Academy of Sciences, Institute of Dendrology, Kornik, Poland.

Suszka, B., Muller, C. and Bonnet-Masimbert, M. (1996). Seeds of forest broadleaves-from harvest to sowing. Institut National de la Recherche Agronomique (INRA), Paris.

Yacine, A., Valero, M. and Bouras, F. (1996). Variation for male success in holm oak, a monoecious and a highly self-incompatible species. *ISHS Acta Horticulturae,* **437**, VII International Symposium on Pollination.