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STORAGE OF CORK OAK (*QUERCUS SUBER* L., 1753) ACORNS
AND EFFECT OF STORAGE DURATION ON SEEDLINGS VIGOUR:
ARTIFICIAL REGENERATION IMPLICATIONS.

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RÉSUMÉ.— *Conservation des glands de Chêne-liège (Quercus suber L., 1753) et effet de la durée de conservation sur la vigueur des plants : implications pour la régénération artificielle de l'espèce.*— Afin de pallier l'irrégularité des glandées de Chêne-liège et de pouvoir faire le semis des glands au bon moment, le recours à la conservation des glands est incontournable. Ainsi, l'objectif de ce travail était de rechercher une technique de conservation permettant de maintenir le plus longtemps possible la viabilité de ces semences récalcitrantes. À cet effet, l'aptitude à la conservation des glands récoltés de deux provenances a été suivie pendant une durée de 21 mois. Après un tri rigoureux, les glands apparaissant sains ont été soumis à trois durées de ressuyage différentes, emballés dans quatre types de contenants et mis en conservation en chambre froide maintenue entre 0 et 2°C. Après chaque trois mois de conservation, 500 glands sont extraits de chaque modalité pour déterminer la teneur en eau des glands, leur pouvoir germinatif et les pertes en glands. En outre, une expérience a été conduite en pépinière avec des glands conservés afin de vérifier s'ils étaient aptes à fournir des plants de qualité suffisante. Les résultats obtenus montrent que les glands de Chêne-liège peuvent être conservés en bon état au moins jusqu'à 21 mois. Il apparaît, par ailleurs, que la durée de conservation dépend de l'origine des glands et du ressuyage subi avant conservation. Les fûts plastiques fermés sont plus appropriés à la conservation des glands que les fûts ouverts et les sachets en polyéthylènes testés dans cette étude. Une teneur en eau initiale des glands comprise entre 37 et 40% est recommandée avant mise en conservation. En outre, les résultats de l'essai conduit en pépinière montrent que les glands conservés plus d'une année dans les fûts fermés fournissent des plants plus vigoureux et que le semis des glands en avril semble propice pour l'élevage des plants en pépinière. Certainement, cette longue durée de bonne conservation connue pour les glands de cette espèce revêt un grand intérêt pratique pour sa régénération, constitue une base pour la conservation à long terme des semences et pour de futures recherches pour expliquer la perte de viabilité des semences récalcitrantes.

SUMMARY.— To palliate the irregular periodicity of *Quercus suber* acorn production and to sow in the appropriate time, the safe storage of seed of the species is unavoidable. Thus, the aim of this work was to find out a storage method that allows to preserve as long as possible the viability of cork oak acorns, already known as recalcitrant seeds. Thus, the storability of cork oak seeds harvested from two seed sources have been followed through a period of 21 months. After a vigorous cleaning, apparently healthy and intact acorns were subjected to three drying periods and stored under low temperature (0-2°C) within four storage containers. Five hundred acorns from each storage combination were sampled at three-month intervals for moisture content determination, germination tests and acorn losses. Additionally, a nursery experiment was carried out with fifteen-month well preserved acorns to evaluate whether or not they were able to develop further into normal seedlings. The results obtained show that cork oak acorns can be safely stored up to twenty-one months without substantial reduction in germinability. Moreover, storage life span seems influenced by seed origin and drying prior to storage. Sealed drums are better for long storage as compared to unsealed drums and sealed bags here tested. An initial moisture content ranging between 37 and 40 % would be suitable for storage success. Furthermore, results got from nursery trials showed that acorns kept inside sealed drums even ones after one-year storage produce more vigorous seedlings and that sowing of acorns in April seems optimal for containerized seedling production. Certainly, this long safe storage period known for this species is of considerable practical implication to its regeneration, set a solid base for longer seed storage and to carry out further investigations to explain viability loss in these recalcitrant seeds.

Seeds that cannot be stored by orthodox means (low moisture content, sub-freezing temperatures) are often called recalcitrant (Bonner & Vozzo, 1987). Seed storage of this group remains an unsolved problem and there is no satisfactory method for maintaining their viability over the long term (Hong *et al.*, 1996; Hong & Ellis, 1996; Iakovoglou *et al.*, 2014), the cause and processes of losing viability is unclear (Cho *et al.*, 2001; Greggains *et al.*, 2000). Their short longevity (Crocker & Barton, 1953) and their limited storage potential are a significant problem in the maintenance of genetic seed banks for long-term conservation (Hendry *et al.*, 1992; Roberts, 1973). Indeed, several factors contribute to the susceptibility for seed deterioration during storage: initial seed quality, storage temperature, moisture content and mycoflora (Malik & Jyoti, 2013). Through the control of the metabolism of seeds, moisture content is one of the most crucial factors in maintaining viability of seeds in storage (Barton, 1961; Bonner, 1981; Connor & Sowa, 2003; Crocker & Barton, 1953; Harrington, 1972; Heit, 1967; Holmes & Buszewicz, 1958; Gosling, 1989; Özbingöl & O'Reilly, 2005; Schroeder & Walker, 1987; Sisman & Delibas, 2005; Sowa & Connor, 2003; Tompsett & Pritchard, 1998; Vashisth & Nagarajan, 2009; Nahar *et al.*, 2009; Wang *et al.*, 2001; Shaban, 2013), particularly for seeds to be stored for long periods and at above freezing temperatures (Barnett, 1969, Huss 1954). The extent to which dehydration can be tolerated varies amongst species (Chin & Robert, 1980) and within species [e.g. *Quercus rubra* (Suszka & Tylkowski, 1980, 1982)]. Beside the difficulties above, for *Quercus suber* acorns exhibiting recalcitrant post-harvest physiology (Roberts *et al.*, 1984; Bonner & Vozzo, 1987; González-Rodríguez *et al.*, 2011; Xia *et al.*, 2012), fungus development, in particular *Ciboria batschiana* (Zopf) Buchwald (*Ascomycetes, Sclerotiniaceae*), is another factor limiting their long-term storage (Merouani *et al.*, 2005). In fact, because of irregular acorn periodicity (personal obs.) and that acorns maturity (November- January) does not correspond with the appropriate nursery sowing time (April), successful method of cork oak acorn long storage is required to ensure a continuous production of planting stock and, consequently, an uninterrupted reforestation program. Regardless its socio-economic and ecological importance for the Mediterranean forest, several works were interested in the regeneration of the species (e.g. Lepoutre, 1968; Messaoudene, 1984, Sondergaard, 1991; Hasnaoui, 1998; Messaoudene *et al.*, 1998; Díaz-Fernandez & Gil Sanchez, 1998; González-Rodríguez *et al.*, 2011), however, contrary to some other oak species [e.g. *Q. rubra* (Suszka & Tylkowski, 1980, 1982)], the storage behaviour of *Q. suber* acorns has received little attention except works realized in Portugal (Merouani *et al.*, 2001, 2005). Therefore, there is an urgent need to carry out more research into the storage of the species. Accordingly, this study is part of successive studies undertaken in the regional station of forest research of Jijel to find the appropriate storage conditions allowing to maintain the viability of cork oak acorns as long as possible.

Besides the common parameters used to assess the efficiency of the storage combinations here experimented, a nursery experiment was carried out with fifteen-month well preserved acorns to evaluate whether or not they were able to develop further into normal seedlings for planting stock.

MATERIAL AND METHODS

SEED COLLECTION AND HANDLING

Acorns used in the experiment were hand-collected in December 2012 from the ground under contiguous trees of the same age (it is realized that the cork oak stands are generally even-aged due to the deficiency of natural regeneration) of two natural cork oak stands localized in Jijel (East of Algeria): coastal cork oak forest of Kissir (36°79' N, 5°66' E) and mountain cork oak forest of Texanna (36° 66' N, 5°78' E). The harvested acorns were placed in plastic boxes and immediately transported to the laboratory where they underwent, within 48 h, a rigorous cleaning conducted in two phases: visual examination to remove extraneous material (impurities, leaves, cupules), immature and insect-infested acorns at first and elimination of small, dried and empty acorns which float (Boland, 1985; Pichon & Guibert, 2001) by flotation test in a

bag filled to 2/3 water, in a second time. After these two operations, two lots of 350 kg of mature, intact and apparently healthy acorns were used for the experimentation.

STORAGE MODALITIES

Although many factors influence the longevity of recalcitrant seeds in storage, only two factors have been considered in the present study: seed treatment prior to storage and storage container.

Storage treatments

After water flotation, a subplot of acorns was dried under laboratory environment for three days (TD), a second subplot of acorns was dried under laboratory environment for one day (PD) and a third subplot of acorns were stored without drying (ND). To encourage uniform drying, the acorns were spread uniformly on a table and mixed regularly (Özbingöl & O'Reilly, 2005). The initial moisture content at the time of storage was determined for each subplot after the drying operation. Thus, the acorns of each subplot were immediately stored after achievement of the drying time.

Storage container

The acorns were stored in five container types:

Sealed plastic drum 80 L capacity allowing a hermetic storage (SD);

Unsealed plastic drum 80 L capacity allowing an open storage (UD);

Sealed clear polyethylene bag 130 µm thick allowing a sub-hermetic storage (TB);

Sealed clear polyethylene bag 10 µm thick allowing a sub-hermetic storage (FB);

Sealed bag of woven polypropylene as a control container (C).

The combination of these parameters allowed establishing 17 storage modalities as reported in Table I.

TABLE I
Storage combinations tested

Storage container	Drying time		
	3 days	1 day	0 day
Sealed plastic drum 80 L capacity	K (V), T (XII)	K (III), T (IX)	K (I), T (VII)
Unsealed plastic drum 80 L capacity	K (VI), T (XIII)	K (IV), T (X)	K (II), T (VIII)
Sealed polyethylene bag 130 µm	T (XV)	-	-
Sealed polyethylene bag 10 µm	T (XVI)	T (XI)	T (XIV)
Sealed bag of woven polypropylene (Control)	-	-	K (XVII)

K: Kissir seed source; T: Texanna seed source

Conditioning and storage environment

In order to prevent an eventual spread of fungus to healthy acorns throughout the storage period, all acorns were coated with a fungicide (Rhodiasan 80 % thiram) before their storage. The acorns were subsequently mixed in dry cork powder and packed in drums or directly packed in polyethylene bags and control bag without cork powder. All storage containers are filled to 80 % capacity. As generally accepted that constant low temperature and relative humidity are necessary for long-term storage of seeds (Arvier, 1983), the storage was conducted in controlled chamber maintained at a temperature oscillating between 0° and 2°C and 75-80 % of relative humidity. Under such low temperature, we believe to reduce the metabolism of acorns without damaging them, to limit therefore their pre- germination and to inhibit also fungal proliferation.

ASSESSMENT OF ACORNS VIABILITY

The efficiency of the storage combinations expressed by the kinetics of moisture content, germination and acorns losses was monitored at regular intervals of three months, the final test being carried out after 21 months of storage. Unfortunately, due to an error during processing, the acorns remaining viable at the 21st month have been involuntarily removed from the cold room.

A working sample of 500 acorns was randomly extracted from each combination at each sampling date to make the following measurements (this large sample seems enough to reduce sampling error for a mixed seed lot). Subsequently, comparison of the results of viability tests after these different acorn's storage combinations (drying status and storage container) should indicate which of the storage combination of those tested minimizes loss in viability during storage.

Moisture content of the cotyledons

Expressed as percentage on a fresh weight basis, water content (MC %) was carried out with a sub-sample of 100 intact acorns among the 500 removed from each storage combination. To determine fresh weight (FW g), the 100 acorns

were weighed separately immediately upon removed from storage (each acorn was identified with a number from 0 to 100). The acorns were then oven dried at 105°C for 24 hours (Justice & Bass, 1978; Gosling, 2002; Özbingöl & O'Reilly, 2005; Reed, 2005), by which time seed dry weight was constant (personal data), and weighed for dry weight (DW g). The individual moisture content was calculated using the following formula:

$$\text{Moisture content (MC) \%} = [(FW-DW)/FW] \times 100$$

The mean moisture content obtained of each sub-sample after storage was compared to the mean moisture content calculated at the time of storage following each dry down.

Germination test and parameters

All germination tests were conducted with a sub-sample of 100 apparently intact acorns among the 500 moved from each storage combination. The acorns were sown in sawdust moistened with water whenever necessary and placed under laboratory conditions. Germination defined as radical protrusion by at least 2 mm (Daws *et al.*, 2006; Snow, 1991; Xia *et al.*, 2012) was scored three times a week for one month. The parameters being considered to evaluate the germinability were germination percentage calculated on the 30th day as the total of germinated acorns and germination speed as the time required to reach a germination of 50 % (Corbineau *et al.*, 2001).

Storage losses

A percentage of losses in acorns during storage was calculated among the 500 moved at quarterly intervals from each storage combination. Losses include pre-germinated acorns, infested acorns and un-germinated acorns. Acorns that failed to germinate by the end of the test period were cut in half using a scalpel and visually examined to determine their condition according to Noland *et al.* (2013): Acorns with no visual cotyledon discoloration were scored in the healthy group; acorns with dark cotyledon discoloration were scored in the fungi-infected (decayed) group and those with brown cotyledon discoloration were considered as dead.

SEEDLING VIGOUR

The goals of seed storage are successful germination and seedling establishment (Hong & Ellis, 1996). To evaluate whether or not well-preserved acorns were potentially able to develop further into normal seedlings, fifteen-months well-preserved acorns of the combinations I, III, V and XII were sown on April 2014 considered as the best sowing period for the species (personal obs.). Eighty intact seed of each combination were sown in open-bottomed containers types WM of Reidacker (17 cm deep with top opening 25 cm² and volume 400 cm³), made of thin plastic and filled with the same growing mixture made of forest oak-humus and cork granules (1:1, v/v). All seeds were sown at the same depth (≤ 1 cm). The containers were arranged in a completely randomized design. Irrigation and control-weeding were uniformly given as and when necessary throughout the vegetation period.

Seedlings height (H) and collar diameter (D) were measured for all surviving six-months old seedlings. Seedling vigour was calculated following Abdul-Baki & Anderson (1973) as:

$$SV = \text{germination (\%)} \times \text{seedling length (cm)}$$

STATISTICAL ANALYSES

The experiment was conducted in a completely randomized design. An analysis of variance was used to compare the moisture content and germination acorns of both storage combinations. When significant differences were found in the ANOVA test $p = 0.05$ probability level, means were separated according to Newman & Keuls multiple range test. Likewise, the growth parameters of seedlings were subjected to one-way analysis of variance employing Newman & Keuls multiple range test at $p = 0.05$. All statistical analyses were performed with the XLSTAT software package.

RESULTS

MOISTURE CONTENT

The data of mean moisture content registered throughout storage are shown in figure 1. Prior to storage, initial moisture content ranged from 36.98 to 40.46 %, showing a slight but not significant ($P < 0.05$) variation, in all cases, within the species and following the three drying periods. Throughout storage, registered moisture content levels appeared affected by storage duration and storage combination.

The acorns stored in sealed drums with an initial moisture content ranging between 37.80 and 40.46% for Kissir seed source (I, III and V) and between 37.03 and 40.06% for those of Texanna (VII, IX and XII), lost respectively 3.19 to 4.37 % and 2.82 to 3.94 % of water during the first three months of storage, except with the acorns of the XII storage method maintained at their initial moisture content. In the sixth month, a decrease of about 5 % of water content was observed

for Kissir acorns and 1-4 % for those of Texanna, except for acorns of the V storage combination for which the moisture content appeared relatively stable. Acorns of Kissir showed a trend to hydration in the ninth month for non-dried acorns (I) and 1-day dried acorns (III), while a decrease of 3 % was recorded for acorns dried for 3 days (V). At this ninth month of storage, a light rehydration of 1.27 % was recorded for acorns of Texanna dried for 3 days (XII), while a decrease of 3 % was recorded for non-dried acorns (VII).

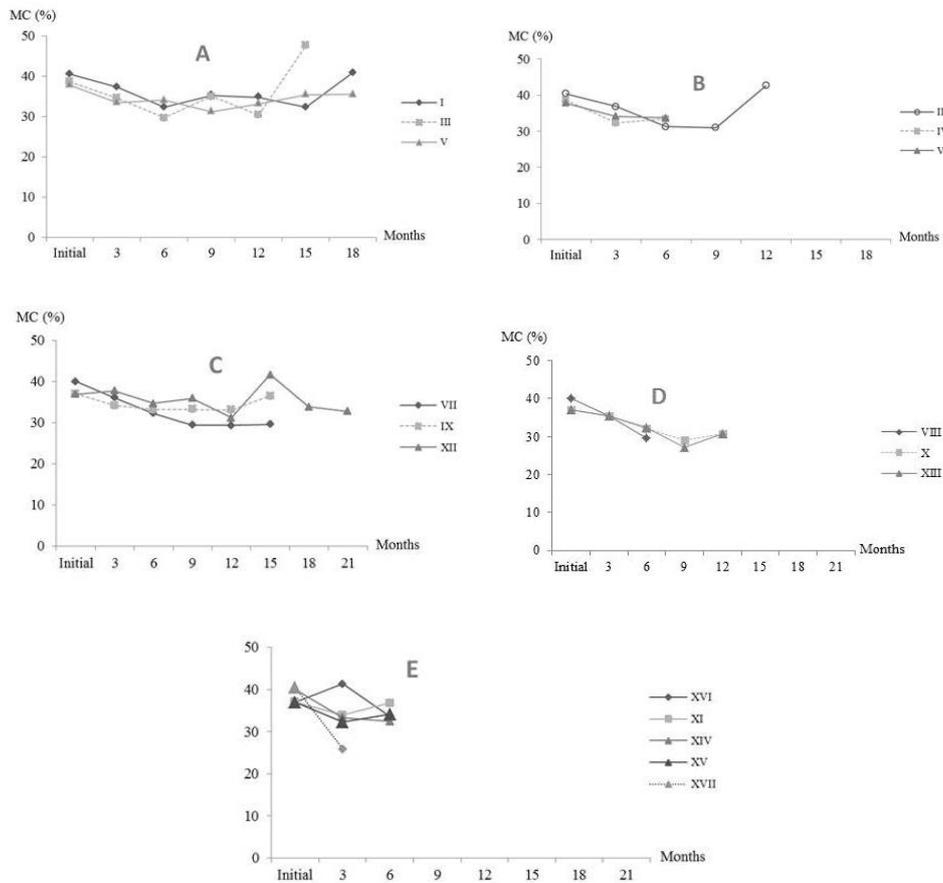


Figure 1.— Changes in mean moisture content according to length and conditions of storage. Each sample point represents the mean of 100 acorns. Drying status: circle = 0 day; triangle = 1 day; square = 3 days. A, B, C, D and E represent respectively acorns of Kissir stored in sealed drums, acorns of Kissir stored in unsealed drums, acorns of Texanna stored in sealed drums, acorns of Texanna stored in unsealed drums and acorns stored in plastic bags and control

After one year in storage, except for acorns of Kissir 3-days dried (V) for which a hydration of 2 % was recorded, water losses from 0.1 to 5 % were recorded for acorns of the other storage combinations. In the fifteenth month, except of 2.5% of water losses registered for acorns of the (I) storage combination, gains in water from 1 % (VII) to 17 % (III) were recorded for the other storage combinations. At the eighteenth month, a different trend of the moisture content was registered: a decrease of 7.76 % was recorded for the acorns of Texanna (XII), while a gain in water of 8.55 % was recorded for acorns of the storage combination (I). In the storage at month twenty-one only remained the acorns of the (XII) storage combination which exhibited a moisture content of 32.80 %.

The moisture contents of acorns stored in unsealed drums fell gradually during the first six months of storage with water losses ranging between 4 and 10 %. While the moisture content kept stable for the acorns of the II storage combination, water losses persisted until the ninth month of storage for the acorns of the combinations X and XIII, relatively well preserved with respective water losses of 3.18 and 5.23 %. After one year of storage, the acorns of the two remaining storage combinations X and XIII shew a trend to hydration with respective gain in water of 1.57 and 3.4 %, while the gain in water was more pronounced with a rate of 11.74 % for the acorns of the combination II. All acorns placed in unsealed drums were completely lost after one year of storage.

Acorns kept in the polyethylene bags 10 μm thick (XI, VIX and XVI) and 130 thick (XV) experienced too an early progressive dehydration over the first six months. The acorns of the control storage combination XVII did not remain more than three months on storage, exhibiting a marked decrease of water content which rapidly reached values lower than 30 %.

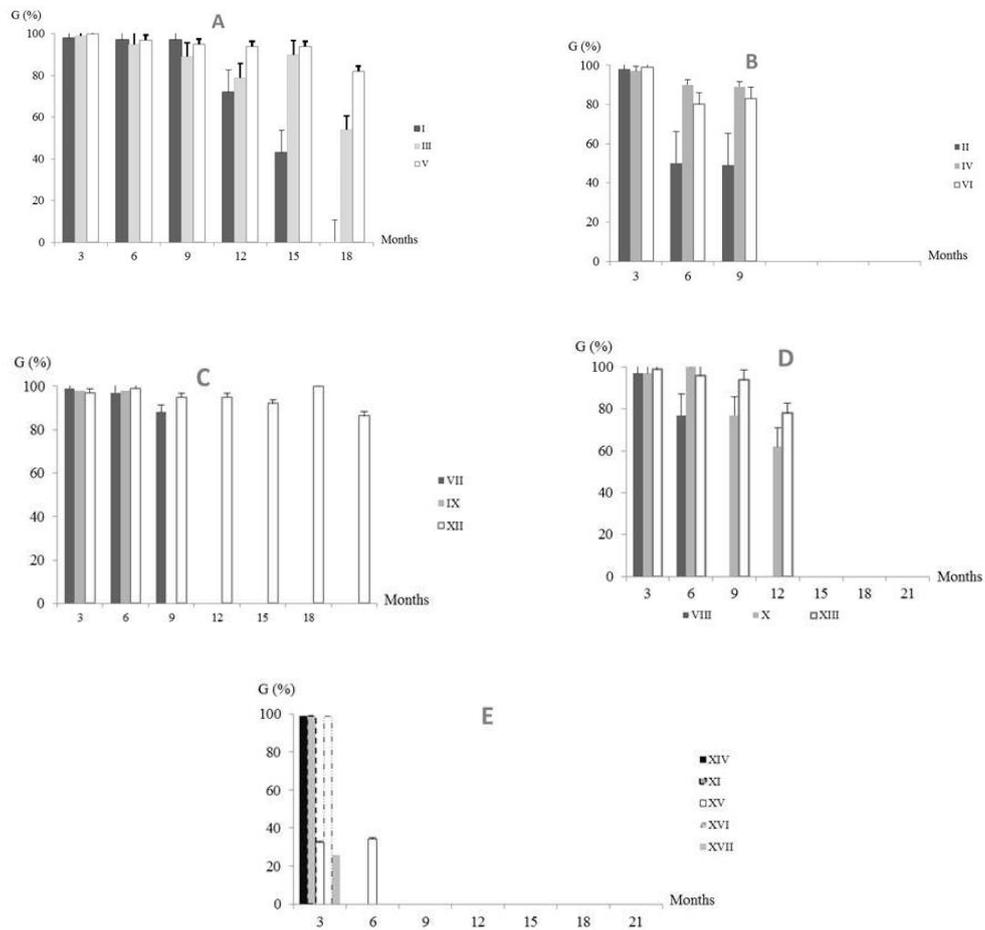


Figure 2.— Changes in germination percentages of acorns according to length and conditions of storage.

The acorns that germinated normally during the germination period were counted and the total number of acorns germinated was divided by the total number of acorns placed in moist sawdust and the result was expressed as percentage. Each bar represents a storage combination and bars of the same colour correspond to the same drying period (black = 3 days, gray = 1 day and white = 0 day).

GERMINATION

Germination percentages registered throughout storage are shown in figure 2. The data recorded about the acorns germination with respect to the storage combinations were similar at 3-months storage. On the whole, with the exception of acorns stored in sealed drums for which the germination percentages remained up to 95 %, germination rates shew a clear decrease for the other storage containers after 6-month storage.

After 9 months of storage, the acorn's germinability shew a decrease for the sealed drums but with satisfactory values exceeding 88 % of germination, while a more significant decrease was registered for acorns stored on some unsealed drums. It appeared in this ninth month that the acorns dried for 3 days (combinations V, XII and XIII) exhibited the highest germination percentages compared with non-dried and one-day dried acorns.

After one year in cold room, the apparently intact acorns stored in sealed drums (VII and IX) failed to germinate, while the germination percentages of the other sealed drums showed a slight decrease to a minimum of 72 %. The acorns stored on some unsealed drums (II, IV, VI and VIII) were totally pre-germinated, whereas others exhibited a germination percentage of 62 % for the combination X and 78 % for the combination XIII.

The acorns of the hermetic combinations I, III, V and XII remained until the fifteenth month all showed satisfactory germinations, reaching percentages of 43, 90, 94 and 92 %, respectively. These germinations were relatively synchronized with respective percentages of 27, 84, 92 and 65 % recorded during the first two weeks of moist stratification.

Acorns stored in sealed drums (combinations III, V and XII) and still viable in the eighteenth month exhibited high germination percentages with respective values of 54, 82 and 100 %. At the end of the experiment, only the acorns of Texanna seed source dried for 3 days and stored in sealed drums remained viable with a high germination percentage of 86.40 % where 80% of acorns have germinated within 15 days of moist stratification.

ACORN LOSSES

The data of table II show that in general the acorns stored in bags XI, XIV, XV, XVI and XVII were rapidly lost before even six months of storage either by early germination (radical exceed 5 cm) or by decay. After one year of storage, early germination percentages of 48 and 51 % were respectively recorded for acorns stored in sealed drums III and XII, except for acorns of the X and XIII storage combinations exhibiting, respectively, losses of 26 and 39 %, while acorns stored in unsealed drums showed losses ranging from 90 to 100 %.

In the fifteenth month of storage, it persisted only acorns stored in sealed drums, including three storage combinations of Kissir seed source (I, III and V) and one storage combination of Texanna seed source (XII). At this experimental stage, a significant proportion of acorns of the storage combinations I, III and V showed broken pericarp which germinated after moist stratification and, therefore, useable for seedling production, however (data not shown).

The acorns still viable at the eighteenth month of storage were those stored in sealed drums III, V and XII, while a total viability loss was registered for the acorns stored in the sealed drum I. Fungi infestation appeared at this stage with rates ranging from 12 to 34 %. At the end of the experiment, there remained viable only acorns of Texanna seed source slowly dried within 3 days and stored in sealed drum XII.

SEEDLING GROWTH AND VIGOUR

The data recorded for seedling growth are shown in figure 3. Analyses of variance showed that seedling growth is influenced by the seed storage combination. In fact a variance difference higher than the set limit ($p = 0.05$) was established for the variables height and collar diameter.

TABLE II

Acorns losses during storage according to storage combinations

Storage combination	Acorns losses(%)																			
	March 2013			June 2013			September 2013			December 2013			March 2014			June 2014			September 2014	
	G (%)	P (%)	DP (%)	G (%)	P (%)	DP (%)	G (%)	P (%)	DP (%)	G (%)	P (%)	DP (%)	G (%)	P (%)	BP (%)	G (%)	P (%)	BP (%)	G (%)	P (%)
I	0	0	0	0	0.4	0	0	0	0	2.4	6.4	0	0	0	65.6	0	34.2	0		
II	23.8	2	1.4	80.8	2.8	0	67.4	0.6	0	83.6	6.4	0	100	0	0					
III	0.6	0.8	0	20.8	0.8	0	59.8	0.2	0	47.8	0	0	0	0	97.0	30.0	15.8	0		
IV	15.4	0.6	2.6	81.8	0	0	72.4	0	4.8	100	0	0	100	0	0					
V	0.8	0	0.8	10.2	0	0	0	0	0	0	0	0	0	0	85.4	38.4	16.0	0		
VI	23.2	2	1.4	81.0	0.6	0	55.0	0	0	100	0	0	100	0	0					
VII	1	0.4	0	2.6	0.2	0	47.2	0.2	0	0	0	0	0	0	0					
VIII	10.6	1.2	1.4	57.8	3.4	0	0	100	0	100	0	0	100	0	0					
IX	0.8	0	0	35.4	1.2	0	0	0	0	0	0	0	0	0	0					
X	16.4	0	0.4	24.2	1	0	22.0	0	2.6	13.75	12.0	0	-	-	-					
XI	27.8	0.2	0	72.8	16.4	0	100	0	0	100	-	-	-	-	-					
XII	0.4	0.2	1.2	33.2	0	1	42.0	1	2.2	51.4	5.4	0	58.52	0	0	37.60	12.38		42.0	1.0
XIII	0.8	0	0.8	42.6	0.4	1	29.2	1.2	4.6	38.6	0.8	0	100	-	-					
XIV	19.81	0	0	88.6	0	0	0	100	0	-	-	-	-	-	-					
XV	29.4	0	0	89.6	0	0	-	100	-	-	-	-	-	-	-					
XVI	20.68	0	0	74.64	5.4	0	-	100	-	-	-	-	-	-	-					
XVII	27.96	0	9.51	0	100	0	-	-	-	-	-	-	-	-	-					

The total number of acorns lost was divided by the 500 acorns removed from each storage combination at each sampling date and the result was expressed as percentage.
 # Losses include four categories: acorns germinated during storage (G), infested acorns (P), acorns with dried pericarp (DP) and acorns with broken pericarp (BP).
 # When losses registered for a storage combination were found superior to 70 % this one was considered as ineffective and automatically removed from the controlled chamber.

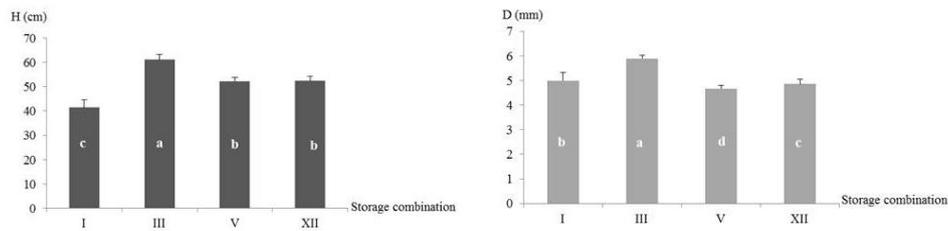


Figure 3.— Mean height and collar diameter with standard errors of cork oak containerized seedlings supplied by acorns stored for fifteen months. Bars with no letter in common differ significantly at 5 % probability level, according to Newman & Keuls multiple range test.

The highest six-month old seedlings were provided by seeds of the storage combination III with an average height of 61.08 cm, followed by those supplied by seeds of the storage combinations XII, V and I with mean heights of 52.38, 52.26 and 40.33 cm, respectively. The six-month-old *Q. suber* seedlings exhibited average diameters ranging between 4.99 and 5.89 mm. The highest diameters were attained by seedlings from seeds of the storage combination III (D = 5.89 mm), followed by those from the combinations I, XII and V with respective mean values of 4.99, 4.86 and 4.67 mm. As regard seedling vigour, the highest index of seedling length was calculated to be about 7027.2 for seedling supplied by seeds of combination III, followed by those supplied by the combinations V, XII and I with respective SV of 6510.44, 6382.96 and 2509.48.

DISCUSSION

MOISTURE CONTENT

The moisture content of cork oak acorns stored in the sixteen storage combinations varied throughout the storage period. Prior to storage, on an average for both storage conditions and combinations, moisture content ranged from 36.98 to 40.46 % but showed a slight variation according to the drying status (Fig.1). In fact, the initial moisture content showed decreasing trend

with respect to the three drying treatments with values of 40.46, 38.60 and 37.80 % registered for undried acorns, 1-day dried acorns and 3-days dried acorns from Kissir seed source, respectively. The same decreasing tendency of initial moisture content was observed for acorns from Texanna seed source with values of 40.06, 37.03 and 36.98 %, respectively registered for undried acorns, 1-day dried acorns and 3-days dried acorns. Under laboratory environment, the acorns have been slowly and partially dried, therefore, the dehydration levels following 1-day and 3-days were not significant at $p = 0.05$. Likewise, highly recalcitrant seeds of *Avicennia marina* showed no reduction in their moisture content after 10 days of drying in a dry air stream (Pammenter *et al.*, 1984). In this context, it has been suggested that mild drying prior to storage could reduce germination and so extend storage life span (King & Roberts, 1980). However, incongruent opinions have been expressed about the faster drying effects on the moisture content at which recalcitrant seeds lose their viability (Berjak & Pammenter, 2008). Other researchers found that viability loss of recalcitrant seeds of *Quercus rubra* (Pritchard, 1991), *Quercus robur* (Finch-Savage *et al.*, 1992) and *Quercus nigra* (Bonner, 1996) was independent of drying rate.

On the other hand, it has been suggested that a lack of drying rate effect could be connected to seed size (Tompsett, 1984; Daws *et al.*, 2004; Berjak & Pammenter, 2008). Indeed, a wide variation in seed size is showed within *Q. suber* species (personal obs.). In this regard, the larger recalcitrant seeds of *Q. suber* studied in the current experiment (especially those of Kissir with a mean weight of 8.46 ± 1.90 g calculated for 1000 acorns), the more slowly will embryo, cotyledon and pericarp dehydration proceed. Moreover, a study on the pericarp micromorphology of *Q. suber* acorns showed that the pericarp, by its thicker and layered microstructure, functions to prevent rapid water loss (Chin *et al.*, 1989; Sobrino-Vesperinas & Viviani, 2000).

In general, the mean initial moisture levels measured in this study are lower than those reported for cork oak acorns in other studies: 41 - 51 % (MC) Merouani *et al.* (2001) for Southern Portuguese seed source (N 38°33', W 8°42') and 48.4 % (MC) by Xia *et al.* (2012) for Italian seed source (N 42°46', E 11°06'). However, the differences may be provenance (habitat from where the seeds were collected) related. These discrepancies of the moisture content levels before storage confirm, obviously, the water content variability within recalcitrant seeds of this species.

The acorns kept in sealed drums all showed fluctuation in moisture content during the storage as revealed by the statistical analysis at $p = 0.05$. Both for acorns of Kissir seed source (I, III and V) and those of Texanna seed source (VII, IX and XII), water losses of 1 to 5 % were registered during the first year of storage with moisture content values, in all cases, superior to 30 %. Trend to hydration was recorded in the fifteenth month for the majority of the storage combinations; the rate of increasing was highest in acorns of the storage combination III (17 %). A different behaviour against the moisture content appeared at the eighteenth month: whereas a decrease of 7.76 % was recorded for the acorns of Texanna (XII), a gain in water of 8.55 % was recorded for acorns of Kissir (I). In the twenty-one months of storage it remained only the acorns of the XII storage combination harvested from Texanna seed source and dried for 3 days with a moisture content of 32.80 %.

As long as the drums are well sealed not allowing, therefore, water exchange with the surrounding air, these fluctuations in water content when measured for the same drum, although weak, confirm that acorns remain metabolically active even under such storage conditions. Moreover, it should be recognized that these variations may result from variations in moisture content among individual seeds since they were harvested from different parent trees. In this regard, Berjak & Pammenter (1997) stated that there are usually marked differences in axis water contents among individual seeds even when harvested simultaneously. Thus, each seed lot must be processed separately. The moisture levels here recorded seems, however, suitable for storage with respect to the results subsequently registered for acorns losses and germination rates.

In open drums, the registered moisture contents fell gradually during the first year of storage. Because of the exchanges with the surrounding environment (RH was near 75-80 % through the

storage period), trends to hydration appear after one year for the three remaining storage combinations II, X and XIII with respective gain in water of 11.74, 1.57 and 3.4. Likewise, acorns of the control method (XVII) and those stored in polyethylene bags 10 μm thickness (XI, VIX and XVI) and 130 thickness (XV) experienced too early a progressive dehydration and thence lost six months of storage.

ACORN GERMINATION

After similar germination percentages registered for all storage combinations at three months storage with high values ranging from 97 to 100 %, the germinability decreased to 50 % and 77 % for the combinations II and VIII, whilst it remained up to 80 % for the other combinations after six months in storage. At the ninth month, the germinability of acorns stored in unsealed drum II decreased much more rapidly to 49 % for an MC level of 31.24 %, the acorns of sealed drums VIII and IX were unable to germinate, while germination percentages ranging from 77 to 97 % were recorded for the other drums. At this storage duration, it appeared that the acorns dried for 3 days (combinations V, XII and XIII) exhibited the highest germination percentages compared with non-dried and one-day dried acorns.

After a last year in cold room, the acorns stored in the sealed drums VII and IX, apparently intact, failed to germinate. With the exception of the acorns dried for 3-days and stored in sealed drums V and XII maintaining, respectively, high germinations of 94 and 95 %, the germinability lowered to 72 % for the sealed drum I filed with non-dried acorns. The acorns of Texanna stored in unsealed drums (X and XIII) exhibited acceptable germination percentages of 62 and 78 %, respectively.

In the fifteenth month, 90, 94 and 92 % of germination were registered for the acorns stored in the sealed drums III, V and XII, respectively. These acorns appeared, moreover, well preserved ever since the synchronic germination reached by the end of the second week of moist stratification with respective values of 84, 92 and 65 %. In contrast, a substantial decrease in germination to 43 % was registered for acorns stored in sealed drum I (26.80 % at the end of the second week of the test), which indicated obviously physiological changes in acorns of this storage combination with increasing in storage. Unfortunately, delayed and lowered germination of acorns of this combination could not be explained with the available data; however, we believe that this progressive decrease of germination can be indicative of progressive vigour loss for Kissir acorns as a result of ageing with increasing in storage.

When the acorns of the sealed combination I were unable to germinate at the eighteenth month, the acorns stored in sealed combinations III, V and XII exhibited respective germination percentages of 54, 82 and 100 %. The acorns of Texanna seed source dried for 3 days and stored in sealed drum XII remained exclusively viable after 21 months of storage with a high germination percentage of 86 %, where 80 % of them had germinated within 15 days of moist stratification. Hence, the rapid and synchronous germination rate registered after this storage duration will be characteristics of vigorous seeds.

ACORN LOSSES

The results got about the acorn losses show that the acorns packed in bags were much more quickly lost compared to acorns stored in sealed and unsealed drums. These early losses in bags are not due to water losses since they have been wholly maintained above an acceptable value of 32 % MC but they are rather ascribed to pre-sprouting during storage with percentages of 79, 89, 90, 75 and 100 % registered for acorns stored in bags XI, XIV, XV, XVI and XVII, respectively. Prematurely germination was also registered in sealed drums but with radicles significantly less long as compared to those in bags and unsealed drums (no radicle exceeded 5 mm). In fact, the tendency of acorns to germinate in the storage container constitutes a real problem in acorn storage. Bonner & Vozzo (1987) stated that acorns have metabolism so rapid that pre-germination

commonly occurs in storage. Thus, germination during storage has the potential to complicate acorn handling, make sowing difficult and increase susceptibility to fungal damage (Bonner, 2003). In further experiment (data not shown) we found, however, that the acorns that germinated during storage developed to normal seedlings when sown with radicles less than 10 mm in length and showing geotropism. In this regard, reducing even more the storage temperature may be a solution to prevent germination during storage, but this way cannot be envisaged only before carrying out frost tolerance and chilling tests that could help in the determination of the lower storage temperature at which *Q. suber* acorns of different provenances can survive without freezing or chilling damage. Nevertheless, the high losses registered indicate that the three plastic bags tested in this study are not appropriate for storage of cork oak acorns even for short duration. In the same way, maintaining drums opened seem not successful for cork oak acorns storage, at least in these experimental conditions. In contrast, acorns stored in sealed drums I, III, V and XII have been well preserved until the 15th month at which time appeared, however, the provenance effect. In fact, a significant proportion of acorns of Kissir seed source (I, III and V) showed broken pericarp but remained usable as long as they germinated once more after moist stratification. At the eighteenth month undried acorns of Kissir seed source (combination I) were unable to germinate. The cutting test demonstrates obviously a brown discoloration of the cotyledons that can be indicative of acorns death. Several studies have been conducted regarding seed deterioration during storage (e.g. Hendry *et al.*, 1992; McDonald, 1999; Smith & Berjak, 1995; Roos, 1989; Walters, 1998; Begum *et al.*, 2013; Shaban, 2013), but the real cause of this irretrievable natural phenomena remains not completely understood. Anyway, it is clear that with increase in storage period, the seed quality parameters decreased with a relative biochemical changes (Begum *et al.*, 2013). In our study, dried status and provenance (genetic features, biochemical composition and rate of ageing) appears to be, at least, the predisposing factors responsible in viability loss of acorns stored in sealed drum I at the eighteenth month. Thus, the obtained results indicated that the acorns of Kissir seed source were significantly more sensitive to the length of storage, as well as to storage conditions, than those harvested from Texanna. Likewise, we have found in a current confirmatory experiment carried out following the best storage conditions established in this research that the moisture content as well as storability registered after fifteen months are highly correlated to the parent tree (data not shown). Anyway, we believe that exposing acorns of various genotypes to accelerated aging test lead to assess changes in vigour during seed storage that can enhance comprehension of the deterioration process.

The acorns still viable at the eighteenth month of storage are those stored in sealed drums III, V and XII, while a total viability loss was registered for the acorns stored in the sealed drum I. Fungi infestation appeared at this stage with values ranging from 12 to 34 %. At the end of the experiment (twenty-one month), there remained viable only acorns of Texanna seed source slowly dried within 3 days and stored in sealed drum (combination XII). Absurdly, an insignificant infestation percentage of 1 % was registered at this last sampling date. In fact, in addition to the protective effect of fungicide, mixing the acorns with cork powder prior to storage separates the acorns from each other that prevent, therefore, the spread of pathogenic fungi throughout storage. Moreover, it can be said that this low pathogenic infestation may be attributed to the good crop production of the harvest year in which we can have a good part of healthy acorns for storage.

Obviously, the above results indicate that cork oak acorns can be stored in sealed plastic drum at low temperature for up to 21 months. Indeed, such conditions allow to reduce acorns' metabolic activity. Moreover, carbon dioxide will replace the oxygen in the remained headspace volume, as long as drums of both storage combinations are not used at their full capacity. In this regard, a most relevant research has shown that in closed system the accumulation of carbon dioxide inhibit respiration (Crocker & Barton, 1957), which can be used advantageously for storing seeds (Justice & Bass, 1978). Likewise, Tytkowski (1977) reported more effective storage of *Q. robur* at high

CO₂ levels than at low ones. Furthermore, Suszka *et al.* (1996) stated that modified environment (high CO₂ and low O₂ concentration) could be responsible for the maintenance of germination in *Quercus cerris*, a recalcitrant species like *Quercus suber* here studied. Bonner (2008) stated that reduction of oxygen levels will slow metabolism and increase longevity of seeds, while Villers *et al.* (2010) stated that a low O₂ and high CO₂ atmosphere had a lethal effect on storage molds. On the other hand, it should be clear that opening and resealing drums and removing 500 acorns at each sampling date will increase the headspace volume of drums and could induce changes in CO₂/O₂ ratio. Therefore, further work is needed to investigate the incidence of such modifications on acorns and their immediate atmosphere as well as the optimal value of this ratio for long term storage.

SEEDLINGS GROWTH AND VIGOUR

Results of fifteen months stored seeds on seedling height, diameter and vigour are statistically different at $p = 0.05$. Seedlings supplied by fifteen months well preserved acorns of the combinations III, V and VII were better developed than those raised from acorns of the storage combinations I in terms of height and collar diameter, although, all seedlings exceeded the standards recommended for this species. Such growths have led to higher vigour index for seedlings supplied by acorns of the combinations III, V and XII compared to those supplied by the acorns of the combination I producing less vigorous seedlings. Indeed, the rapid and synchronous germination rate registered after fifteen months in storage for acorns stored inside combinations III, V and XII will be characteristics of vigorous seedlings. These observations suggested, however, that there is no substantial, or at least less, decline in seed vigour after fifteen months on storage, and that the sowing in April seems to be the best sowing period for the species. Obviously, these findings confirm the favourable storage conditions experimented in this study for cork oak acorns that enable to preserve seed vigour on the acceptable level for production purposes. In this regard, Clor *et al.* (1976) stated that the storage at low temperature delays oxidative losses of food reserves in seeds preserving, therefore, the ability of embryos to produce vigorous seedlings. In contrast, the lower vigour index registered for the seedlings supplied by acorns of the storage combination I may be related to reserves depletion due to acorns ageing with increasing storage.

IMPLICATIONS TO ARTIFICIAL REGENERATION OF THE SPECIES

The primary purpose of storing seeds is to have a viable seed supply once it is needed for regeneration either by direct seeding or planting after nursery cultivation. Storage of seeds is of paramount importance to preserve the seeds from collection to the time of sowing and to make them available for the years when little or no seed has been produced (Holmes & Buszewicz, 1958). Hence, successful seed storage will allow to overcome fluctuations in seed production that guarantees a continuous reforestation or regeneration program. With exceptions due to provenance effect, our results indicate that fresh acorns of *Q. suber* can be safely stored for at least 21 months without substantial reduction in viability. These findings are of considerable practical implication to *Q. suber* storage and seedlings cultivation. Indeed, to palliate the irregular acorn periodicity, which has, already known, for this species, nursery manager can collect acorns during an abundant seed year and store them, according to the best storage conditions established in this study, until nursery sow over two springs after harvest. If successful, it will be possible to extend the *Q. suber* acorn supply around two years to ensure a continuous production of planting stock. On the contrary, if suitable storage conditions aren't supplied, acorns losses increase significantly, that perturb regeneration program. Moreover, in further experiment (data not shown), we found that the acorns that germinated during storage were still viable after they were removed from storage, and therefore, could be used for normal seedling production if their radicles are less than about 10 mm in length. Moreover, the pruning of the sprouted radicles of up to 10 mm in length prior nursery

sowing does not prohibit seedling production. In fact, in agreement with previous works (Appleton, 1995; Bonner, 1982, 2003; Castle, 1983; Chouial, 2004; Devine *et al.*, 2009; McCreary, 1996), we have observed in further experiment new growth from the ends of broken *Q. suber* radicles, and that the newly formed roots exhibited normal growth and seedlings had a root morphology characterized by multiple taproots. Hence, root surface area becomes larger with seedlings of multiple taproots as compared to seedlings with a single taproot that could enhance seedling field performance especially under harsh site conditions associated to Mediterranean environment. Besides the opportunities above presented, the safe storage offers the opportunity to use the best seeds, to sow in the appropriate time, to have a synchronous acorn germination and homogenous seedling emergence that enhance seedlings uniformity and quality.

CONCLUSION

To palliate the irregular periodicity of acorns production and to sow in the appropriate time, the safe storage of *Q. suber* acorns is unavoidable. The results here obtained show that cork oak acorns can be safely stored over up twenty-one months without substantial reduction in germinability. Moreover, the following conclusions may be drawn from the above results:

- Under the storage environment here supplied, the acorns stored in sealed plastic drums retained better viability throughout the storage period as compared to unsealed drums, polyethylene and woven polypropylene bags here tested.

- Under the same handling and storage conditions, seeds of various provenances of *Q. suber* species lose viability to different extents. Hence, seeds of each provenance must be processed and stored separately.

- The lower values for seed infestation registered proved that after vigorous cleaning, coating acorns with fungicide and mixing them with cork powder prior to storage seems highly effective against fungal proliferation already registered during storage.

- An initial moisture content ranging between 37 and 40 % would be suitable for *Q. suber* acorns storage.

- As recalcitrant seeds, *Quercus suber* acorns can retain viability around two years at near freezing temperature ranging from 0 to 2°C.

- Spring sowing (April) seems to be the optimal period for *Q. oak* containerized seedling production in nursery.

Thus, for successful storage, fully mature cork oak acorns collected from high cropping trees inside a good seed year should be cleaned as soon as possible by visual examination and water flotation and then dried under ambient laboratory environment until reaching 37 to 40% of moisture content, and promptly coated with a non-penetrating systemic fungicide, mixed with dry cork powder, packed in sealed plastic drums filled at 80 % capacity, hermetically sealed and placed in cold room maintained at 0 – 2 C. The drums should be opened for few times once or twice per quarter to adjust the atmosphere (CO₂/O₂ ratio) and to mix acorns at the time.

Certainly, this longer safe storage period known for this species is of considerable practical implication to its regeneration, set a solid base for longer seed storage and to carry out further investigations to explain viability loss in these recalcitrant seeds. However, further researches are required for relationship storability - provenance /parent tree, seed survival under diverse factorial combinations of low temperature - moisture content and optimum ratio CO₂/O₂ in order to find the optimum storage requirements and to maximize even more longevity in storage.

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